INTERACTIONS BETWEEN THE MEDIAL PREOPTIC NUCLEUS AND MESOLIMBIC REWARD PATHWAY IN THE REGULATION OF SOCIOSEXUAL BEHAVIORS

by

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Abstract

A major challenge facing members of most vertebrate animal species is to evaluate the intricate social, physiological, and environmental information they encounter and integrate this information to display the appropriate social behaviors in an adaptive manner. In the case of sociosexual encounters, different brain mechanisms interact to orchestrate information about the salience of the external stimuli along with the physiological and environmental conditions and incorporate them to be able to display fitting sexual behaviors. For all vertebrate species, the medial preoptic nucleus (POM) is a key integrative site in the regulation of male sexual behavior. It is responsive to the hormonal milieu and thus to key aspects of the physiological and environmental conditions. On the other hand, the mesolimbic dopamine system plays an important role in the attribution of incentive salience to sexually relevant cues. There is strong theoretical reasoning as well as experimental evidence suggesting a potential interplay between medial preoptic nucleus and mesolimbic system, however there has been a paucity of studies investigating this interactions and its regulation of male sexual behaviors. This dissertation sets out to elucidate how these two systems communicate with each other to modulate male sexual behaviors based on studies in Japanese quail (*Coturnix japonica*). Quail have emerged as an excellent species in which to investigate the neuroendocrine regulation of male-typical sexual motivation and performance. To this goal, (1) first we demonstrate that both POM and parts of the mesolimbic system are implicated in appetitive and consummatory sexual behaviors via immediate early gene studies. (2) Subsequently we establish an anatomical location for nucleus accumbens in avian species by employing a variety of immunohistochemical and hodological markers

to examine homologies with mammalian species. This investigation enabled us to explore further the functions of nucleus accumbens in the control sexual behaviors and again we demonstrated the involvement of this nucleus in regulation of sexual behavior in both males and females based on immediate early gene expression measures. (3) Subsequently, studies employing the lesion of dopaminergic inputs to the POM and Ac demonstrated that dopamine function in POM and Ac is necessary for the initiation of appetitive and consummatory sexual behaviors. (4) This study was followed by an experiment utilizing an asymmetrical inactivation strategy that focused on the POM and VTA. Our findings provide novel evidence for interplay between POM and VTA in modulation of appetitive but not consummatory sexual behaviors. (5) To investigate how this interaction is implemented we combined tract-tracing methods with immediate early genes and demonstrated that efferent projections from POM to VTA are implicated in sexual behaviors. Overall, this series of experiments demonstrates that appetitive aspects of male sexual behavior are regulated via interplay of POM and VTA. These findings provide novel insight as to how organisms evaluate complex social, physiological, and environmental inputs when processing stimuli with high value in a sexual context.

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To paraphrase Isaac Newton, "*if I have seen further it is by standing on the shoulders of giants*". If this is the case, then we have to praise our mentors for giving us the footholds needed to stand upon the shoulders of those giants, and for instructing us where to set our gaze once we have climbed atop. Thus, first and foremost, I want to thank my advisor Gregory Francis Ball whom has gave me the scientific guidance and freedom, and enabled me to spread my wings in his laboratory. I am proud to have learned from him, and so incredibly lucky to have worked alongside him for these last five years. His unique perspective and deep knowledge of science will always guide me in my forthcoming scientific endeavors. Without a doubt, I also should thank my previous mentors, Professors Hasan Bahcekapili and Resit Canbeyli, whom kindled my scientific interest, without them I would never even imagine to pursue a career in science. In addition, I thank all my thesis committee members for their time and support in completion of my dissertation. Especially, Professor Peter Holland and Professor Shreesh Mysore provided me with much needed advice and support throughout my dissertation process.

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Outdoors all afternoon

under a gunmetal sky

staking my garden down,

I kneeled to the crickets trilling

underfoot as if about

to burst from their crusty shells;

and like a child again

marveled to hear so clear

and brave a music pour

from such a small machine.

What makes the engine go?

Desire, desire, desire.

Stanley Kunitz

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Chapter I - Interactions between Medial Preoptic Nucleus & Mesolimbic Reward Pathway in the Regulation of Sociosexual Behaviors

Sociosexual interactions are essential for reproductive success in sexually reproducing species, and under natural conditions, the occurrence of these interactions comes with intricate challenges and opportunities for animals. Given that these interactions are necessary for individuals reproductive fitness reward mechanisms have evolved to drive males and females to seek out and mate with appropriate conspecifics (Insel & Fernald, 2004; O'Connell & Hofmann, 2012). In addition, depending on the specific context in which these social behaviors occur, we would expect animals to be in different motivational states and to approach the opportunity for sexual interactions in flexible ways (Ball & Balthazart, 2002; Fernald, 2012; Robinson, Mocsary, & Camp, 1994). For instance, in the case of male sexual behavior, costs and benefits of mating behavior are well understood. Reproductive behaviors increase energy expenditure as well as risk of predation and disease; thus, coordination of these behaviors in relation to physical and social cues is critical (Adkins-Regan, 2005). In this context, animals should assess the salience of the external stimuli along with the physiological and environmental information and incorporate them to be able to display fitting sexual behaviors.

In order to produce appropriate behaviors in relation to these complex external and internal conditions, specialized neural mechanisms have evolved to play diverse roles in regulating these responses. Recently, a number of behavioral biologists have argued that two major neural systems, the social behavior network and mesolimbic reward circuitry, interact in the regulation of a wide range of social behaviors (reviewed in Hofmann et al., 2014; Goodson & Kingsbury, 2013; Goodson, 2005; O'Connell & Hofmann, 2011). For male sexual behavior, a component of the social behavior network, the medial preoptic area (mPOA), is considered to

have special importance (Dominguez & Hull, 2005).The mPOA receives inputs from all sensory modalities and sends projections to motor pathways; moreover, it is responsive to hormonal milieu of the organism (Will et al., 2014) . In addition, the mesolimbic system has also been associated with rewarding properties of sexual behavior (Pfaus, 2009). There is strong theoretical reasoning and experimental evidence suggesting a potential interaction between mPOA and mesolimbic system, which has not gone unnoticed by other researchers:

> "*To this date, no one has shown that the mPOA is linked to the mesolimbic DA system in the control of appetitive male sexual motivation. … it is highly likely that future research will confirm that mPOA interactions with the mesolimbic DA system regulate the appetitive aspects of sexual behavior*" (Stolzenberg & Numan, 2011, p. 838).

The present dissertation sets out to explore the potential interplay of medial preoptic nucleus POM (analogue of mammalian mPOA) and mesolimbic system in male sexual behaviors of Japanese quail. In what follows in this chapter, I will present the relevant background information and experimental evidence to support the hypothesis concerning the importance of mPOA – mesolimbic system connections. First, I will describe appetitive and consummatory sexual behaviors. This will be followed by a review of the research findings related to the role of mPOA, and its avian homolog medial Preoptic nucleus (POM), dopamine, and mesolimbic system in regulation of male-typical sexual behaviors. Finally, the evidence for possible POM-mesolimbic system interaction will be discussed. Drawing on this experimental evidence, I will describe a series of experiments, which explore the neural correlates of this putative interplay.

I. Appetitive and Consummatory Sexual Behaviors of Japanese Quail

Japanese quail (*Coturnix japonica*) have attracted considerable attention as a useful animal model for many fields of study, such as behavioral neuroendocrinology, reproductive and developmental biology, and learning (Ball & Balthazart, 2010; Mills, Crawford, Domjan, & Faure, 1997; Ruparelia, Simkin, & Salgado, 2014). General characteristics that make Japanese quail a valuable model are its relatively small size, high egg productivity, low cost, and significantly shorter time to maturity. On the other hand, their extraordinary motivation to engage in sexual behaviors, which are easily quantifiable, makes them specifically useful for our purposes here. In addition, basic research on male sexual behavior in quail has a long history (Adkins & Alder, 1972; Beach & Inman, 1965; Sachs, 1967), and so there is a large body of background knowledge that establishes the groundwork for new studies investigating the underlying neural mechanisms (reviewed in Ball and Balthazart 2010).

In ethological tradition, animal behaviors have been categorized into appetitive and consummatory phases to differentiate goal-directed actions and the stereotypical concluding acts of the goal-directed behaviors. This behavioral dichotomy was first suggested by Charles S. Sherrington (1906), though the terms 'appetitive' and 'consummatory' were coined by Wallace Craig (Craig, 1918) and have been widely used by ethologists throughout the 20th century (e.g., Tinbergen, 1951). This terminology has also been adopted in the study of sexual behavior (Beach, 1956). The usage of these concepts in relation to sexual behavior has been criticized by some researchers (Sachs, 2007), because it can be difficult to establish the exact boundaries between appetitive and consummatory sexual behaviors (Pfaus, Kippin, & Coria-Avila, 2003); even still, this distinction is considered valuable by many researchers (Ball & Balthazart, 2008; Stolzenberg & Numan, 2011), and hormonal and neuronal manipulations do differentially affect behaviors that divide into these two categories (Ball & Balthazart, 2010; Balthazart & Ball,

1998; Everitt, 1990). The present dissertation has also aimed to explore how these two different categories of sexual behavior are implemented differently in the brain.

For sexual behavior, appetitive behaviors can be categorized by the search, identification, and approach of a potential sexual partner, whereas consummatory behaviors consist of actual sexual contact and associated copulatory responses (Pfaus, 2009). In male Japanese quail, consummatory behaviors consist of a stereotypic sequence of the neck grab, mounting, and cloacal contact movements (Adkins & Alder, 1972).

There are two classical courtship behaviors that could be categorized under appetitive sexual behaviors: crowing and strutting; both function to attract potential mates for quail (Adkins & Alder, 1972; Ottinger, Schleidt & Russek, 1982). These two naturally occurring behaviors are good examples of male appetitive sexual behavior; however, since their execution is variable under similar conditions, more quantifiable measures of appetitive sexual behaviors have been developed in male Japanese quail. Studies demonstrated that, after a single copulation, a male Japanese quail will spend most of its waking hours looking through a small window when a female is present (Domjan & Hall, 1986). A modified version of this social proximity response was employed to illustrate how testosterone and dopamine exerts its effects on appetitive behavior (Balthazart et al., 1997). Another type of response commonly used by researchers to be indicative of appetitive sexual behavior is Rhythmic Cloacal Sphincter Movements (RCSM), this behavior occurs in the anticipation of copulation, and increases foam production, which contains sperm, thus increasing the possibility of fertilization (Seiwert & Adkins-Regan, 1998).

Female Japanese quail usually do not show Rhythmic Cloacal Sphincter Movements in response to the anticipation of copulation under natural conditions. However, if adult female quail treated with male-typical concentrations of testosterone, they start to exhibit this behavior

when provided with visual access to male quail (Adkins-Regan & Leung, 2006). Females display infrequent Female-typical Cloacal Sphincter Movements (FCSM) after copulation. Initially, it was hypothesized that these movements, similar to males, enhance the probability of fertilization; however, no direct relation between these movements and fertilization in females has been found (Adkins-Regan & Leung, 2006). Overall, there are limited numbers of paradigms that would assess the appetitive sexual behaviors for female Japanese quail. Though we know about certain factors that may influence female appetitive sexual behavior in quail, such as in mate choice procedures (Persaud & Galef, 2003; White & Galef, 1999), much less is known about the neural control of these behaviors in females. This bias toward male studies in laboratory animals has been subject to criticism in the current literature (Beery & Zucker, 2011), and US National Institutes of Health (NIH) also suggested that this bias should be eliminated (Clayton & Collins, 2014). We take into account these concerns and investigate the brain structures that involved in appetitive and consummatory sexual behavior in chapter 2 and test a novel paradigm to evaluate sexual motivation of female quail in Chapter 3. However the primary locus of the present dissertation is not to establish the sex differences, thus work in the subsequent chapters focus primarily on male quail.

II. Medial Preoptic Nucleus and Sexual Behavior

Medial preoptic area, including the medial preoptic nucleus, has been credited to have a central role in male sexual behavior in vertebrates (reviewed in Hull, 2011). Evidence supporting the critical role of mPOA in male sexual behavior comes from mainly four different lines of research; (1) ablation of the nucleus, (2) stimulation and inhibition of the nucleus, (3) correlates of sexual behavior induced activity in the brain, and (4) direct hormonal manipulations to the nucleus.

Electrolytic lesions to mPOA impairs sexual behaviors in males in a large variety of vertebrates including rodents (Larsson & Heimer, 1964) , dogs (Hart & Ladewig, 1979; Hart, 1974), cats (Hart, Haugen, & Peterson, 1973), goats (Hart, 1986), birds (Bailhache, Surlemont, & Balthazart, 1993), lizards (Wheeler & Crews, 1978) , and fish (Macey, Pickford, & Peter, 1974), but these effects are not limited to the species mentioned here (reviewed in: Hull, Meisel & Sachs, 2002). This preservation of function suggests that the role of mPOA in male vertebrate brain and sexual behavior is evolutionarily conserved and has been supporting males in their ability to copulate for more than 400 million years.

In addition to these early ablation studies, it has been shown that electrical stimulation of mPOA augments male sexual behaviors in rats (Rodriguez-Manzo, Pellicer, & Larsson, 2000). The electrical recordings from mPOA suggested sexual arousal and performance enhanced the firing rates of neurons in rats (Shimura, Yamamoto, & Shimokochi, 1994). Furthermore, an increase in immediate early gene immunoreactivity has frequently been documented in the mPOA in association with consummatory sexual behaviors (Hamson & Watson, 2004; Heeb & Yahr, 1996; Portillo & Paredes, 2004; Robertson et al., 1991; Wersinger & Baum, 1997).

The role of POM for the species of interest in this dissertation, Japanese quail, has also been well documented. Converging evidence from different lines of research including immediate early gene studies (Charlier et al., 2005; Iyilikci et al., 2014; Meddle et al., 1999), stereotaxic injections of aromatase inhibitors (Balthazart, Evrard, & Surlemont, 1990), and testosterone implants (Balthazart & Surlemont, 1990) demonstrate that POM is a fundamental structure in control of sexual behaviors in male Japanese quail (reviewed in Ball & Balthazart, 2004, 2010).

In rats, mPOA has bidirectional connections with all sensory modalities and sends projections to motor pathways; the hodology of the nucleus implies that mPOA is a key brain structure, responsible for the incorporation of information and modulating the motor outputs related to sexual behavior (Simerly & Swanson, 1986, 1988). For many mammalian species, especially for rodents, olfactory cues are the primary access to their umwelt for sexual cues, whereas, for avian species, traditionally visual and auditory information have been considered to have a more central role (Powers & Winans, 1975; for a review see: Keverne, 2004; Petrulis, 2013). For example, in male quail, visual cues alone such as those provided by taxidermic models are sufficient to induce sexual responses in the absence of any auditory and olfactory inputs (Nash, Domjan, & Askins, 1989). Tracing studies have reported sparse projections from hyperpallium accessorium (HA) to POM and is considered as a potential pathway for visual inputs (Balthazar & Absil, 1997). There are no observations that would indicate that auditory inputs play any role in sexual behaviors of the male Japanese quail; consequently, there are no hodological studies that would investigate these inputs. However, for female Japanese quail, there are some studies suggesting that auditory inputs may play a role in mate selection; for example phonotaxis behavior, approach towards audio stimulus, has been reported in response to male conspecific vocalizations (Goodson & Adkins-Regan, 1997). In addition, these vocalizations, called crows, induce ovarian development (Guyomarc'h & Guyomarc'h, 1989). But, as in their male conspecifics, there is a paucity of studies in investigating these inputs. For ring doves, a functional connectivity between auditory thalamus and POM has been established (Cheng, Peng, & Johnson, 1998; Cheng & Zuo, 1994), which suggests a similar input may be involved for female Japanese quail.

Due to early naturalistic observations and lack of rigorous scientific inquiry, avian species were considered to be microsmatic for many years (reviewed in Balthazart & Taziaux, 2009); however, recent findings indicate that interruption of olfactory inputs decreases immediate early gene activation associated with sexual behavior, although it does not affect the sexual behavior per se (Taziaux et al., 2008). In mammals, the olfactory information originating from vomeronasal organ reaches mPOA via the bed nucleus of stria terminalis (BNST) and amygdala (Kevetter & Winans, 1981). In Japanese quail, POM, BNST, and the medial amygdala's homolog, nucleus taenia of the amygdala (TnA), are also bidirectionally connected with one another (Balthazar & Absil, 1997). Based on these projections, it has been suggested that olfactory information in Japanese quail may reach POM through BNST and TnA as is the case in mammals (Ball & Balthazart, 2004).

POM incorporates sensory information, converging via afore mentioned pathways, and regulates motor outputs related to sexual behavior. In mammals, there is strong evidence indicating that projections from mPOA to PAG are the primary motor output for sexual behavior. For instance, electrical stimulation of mPOA elicits urethrogenital reflexes (Sato & Christ, 2000). Furthermore, lesions of PAG disrupt the effects of mPOA stimulation, indicating mPOA-induced activations relay in PAG (Marson, 2004). PAG sends projections to nucleus paragigantocellularis (nPGi), which is the source of inhibition of ejaculatory mechanisms in male rats (Marson & McKenna, 1990; Murphy & Hoffman, 2001).

Similar to mammals, projections from POM to PAG have been documented in avian species by tract tracing studies (Absil, Riters, & Balthazart, 2001; Carere, Ball, & Balthazart, 2007). Furthermore, anterograde and retrograde tracing established descending projections from PAG to the cloacal sphincter muscle through a premotor brainstem nucleus, nucleus

retroambigualis (RAm). (Wild & Balthazart, 2013). Refer to Figure 1 for representative diagrams of neural circuits illustrating afferent and efferent hodology of POM in Japanese quail.

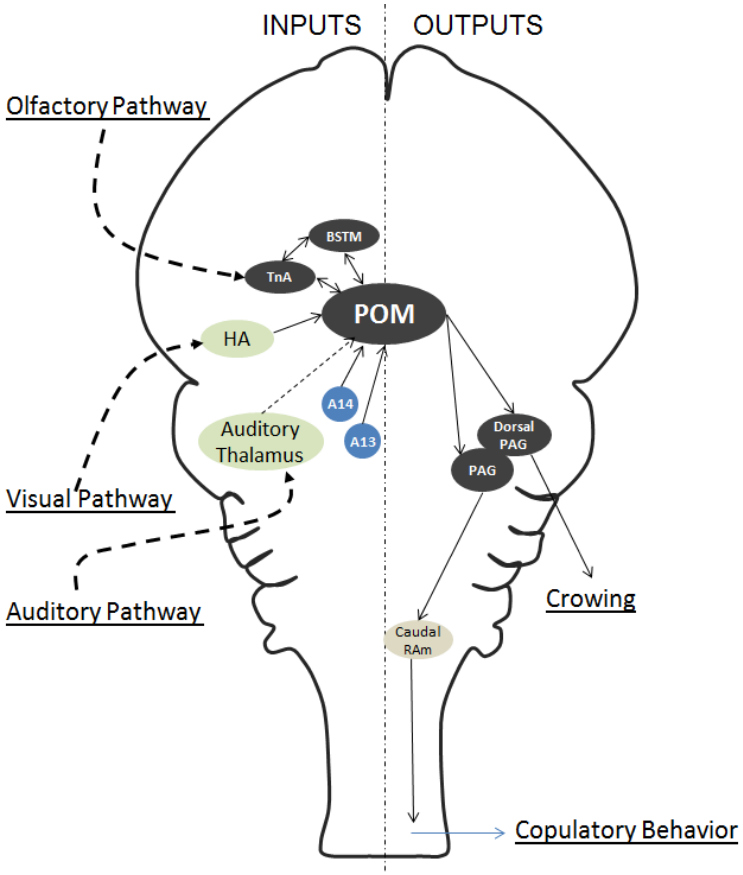

Figure 1. Schematic representation of afferent and efferent hodology of medial Preoptic nucleus of Japanese quail. Solid lines define the documented projections in the Japanese quail brain. Dashed lines indicate presumptive projections based on the work on different species.

POM is also a major site of hormone actions associated with the regulation of sexual behavior (Balthazart et al, 2003). The volume of the nucleus is testosterone-sensitive, and it is larger in males (Viglietti-Panzica et al., 1986). In addition, testicular castration regresses the volume of the nucleus, whereas subsequent testosterone treatment restores it to typical levels (Panzica et al., 1987). The testosterone effects on the structure of POM likely facilitate function

effects as well: testosterone implants targeted to POM restore the sexual behaviors subsequent to castrations (Balthazart & Surlemont, 1990).

Early evidence suggested that different metabolites of testosterone seem to play distinct roles in appetite and consummatory sexual behaviors. Following castration, treating animals with a combination of two metabolites of testosterone, dihydrotestosterone and estradiol, completely recovers appetitive and consummatory sexual behaviors in male Japanese quail. However, when these two metabolites are administered individually, dihydrotestosterone partially activated strutting behavior but not consummatory sexual behaviors, whereas estradiol administrations induced consummatory sexual behaviors but not strutting (Adkins & Pniewski, 1978). Even though dihydrotestosterone may have a role in some appetitive sexual behaviors, recent evidence suggests that aromatization of testosterone (T) to estradiol is critical for both appetitive and consummatory sexual behaviors (Cornil et al., 2006). For example, aromatase inhibitor disrupts sexually motivated rhythmic cloacal sphincter movements, a correlate of appetitive sexual behavior in male Japanese quail (Taziaux, Cornil & Balthazart, 2004). In addition, testosterone implants targeting the preoptic area of male Japanese quail rescues castration-related deficits in consummatory sexual behavior, but only if it is not combined with aromatase inhibitors (Watson & Adkins-Regan, 1989).

III. Dopamine and Male Sexual Behavior

The initial observation about dopamine's facilitatory role in the sexual behavior was made when L-dopa (3,4-dihydroxy-L-phenylalanine), the precursor to catecholamines, treatment used in Parkinson's patients resulted in increased sexual motivation (Woert, 1971). Subsequent experimental studies also supported this observation; L-dopa and apomorphine, a non-selective dopamine agonist, decreased the ejaculation threshold and accelerated the achievement of

ejaculation (Paglietti, Quarantotti, Mereu, & Gessa, 1978), whereas systemic administrations of dopamine receptor antagonists (haloperidol and pimozide) delayed initiation of copulatory behaviors and reduced the number of intromissions (Pfaus & Phillips, 1989).

Over the last decades there is a surging interest on the role of dopamine in sexual behavior (reviewed in; Dominguez & Hull, 2014; Giuliano & Allard, 2001; Hull et al., 2004). However the proximate mechanisms of dopamine in relation to sexual behavior have still not been established entirely. One theory suggests that release of dopamine in specific brain structures removes tonic GABAergic inhibition, thereby disinhibition via DA release increases neuronal excitability resulting with enhancement of sensory-motor integration (Chevalier & Deniau, 1990; Dominguez & Hull, 2005).

Dopamine exerts its effects in sexual behavior via three different dopamine systems: (1) DA in nigrostriatal system contributes to motor initiation and coordination. (2) Da in mesolimbic system is implicated in motivational aspects of sexual behavior. And, (3) DA in incertohypothalamic system participates in initiation of consummatory sexual behaviors and sexual motivation (reviewed in: Giuliano & Allard, 2001; Hull et al., 2004). More specific findings in relation to mesolimbic and incertohypothalamic will be discussed below; however the possible involvement of nigrostriatal DA system is not within the scope of this dissertation.

The mPOA receives afferent projections from different incertohypothalamic DA cell groups including periventricular hypothalamus (A14), zona incerta (A13), and also it receives some projections from midbrain ventral tegmental area (A10). Nevertheless, dopaminergic inputs originating from A14 are considered to be the primary source of dopamine within the mPOA of rats (Giuliano & Allard, 2001). In Japanese quail, dopaminergic afferent projections from A14

and A10 to POM were also been documented (Balthazart & Absil, 1997). Direct infusions of apomorphine, a non-selective dopamine agonist, to the mPOA increased the number of ejaculations in rats (Hull et al., 1986) , whereas, administration of haloperidol, dopamine receptor antagonists, impaired consummatory sexual behaviors (Pfaus & Phillips, 1991). In vivo microdialysis studies in male rats showed an increase in DA levels within mPOA when an estrous female was present, which was interrelated to testosterone levels of the rats (Hull et al., 1995). Likewise, in male Japanese quail, extracellular dopamine levels increased in the presence of a conspecific female, furthermore, this precopulatory increase in dopamine was not present in quail that did not engage in consummatory behaviors (Kleitz-Nelson, Dominguez, & Ball, 2010; Kleitz-Nelson et al., 2010). These similarities in the DA function within hypothalamus between rats and quail suggests a phylogenetically conserved male sexual behavior system (Pfaus, 2010).Overall, these data indicates DA release in POM associated with both consummatory and appetitive aspects of sexual behavior.

Another system implicated in sexual behavior is mesolimbic dopamine system which consists of dopaminergic projections originating from ventral tegmental area to nucleus accumbens. For instance, in vivo microdialysis studies found an increase in extracellular levels of dopamine in nucleus accumbens of copulating male rats in comparison to other locomotor activities (Damsma, Pfaus, Wenkstern, Phillips, & Fibiger, 1992). In addition, both sexual behavior and sex-related environmental cues enhanced immediate early gene reactivity in dopaminergic VTA neurons and nucleus accumbens neurons (Balfour, Yu, & Coolen, 2004).

Mesolimbic system plays an important role in reward processing by modulating the attribution of incentive salience to rewards and cues associated with rewards (Kelley & Berridge, 2002). As in other naturally rewarding stimuli, or addictive substances that hijack this circuitry,

mesolimbic system makes sexual behavior related cues attractive and attention grabbing and initiates a 'wanting' state (Berridge, 2004). For example, in rats 6-hydroxydopamine (6-OHDA) lesions that cause dopamine depletion in the nuclues accumbens reduced the sexual arousal related to the remote cues from females (Liu, Sachs, & Salamone, 1998).

IV. Interactions between the Medial Preoptic Nucleus and the Mesolimbic Reward Pathway in the Regulation of Sociosexual Behaviors

Neuroethological research over the last three decades focused on two different brain mechanisms that may play a critical role in the manifestation of several social and adaptive behaviors in all vertebrates. (1) Studies on behavioral neuroendocrinology predominantly focused on the brain regions that have steroid receptors with an emphasis on the temporal regulation, development, and sexual differentiation of these behaviors (2) research on motivational behaviors focused on the incentive salience of an external stimulus as well as the hedonic responses subsequent to the consummatory behaviors and therefore, the mesolimbic reward circuitry became a primary target of their investigation. These two different interests in study of same naturally rewarding behaviors differ in their approach and priorities, consequently two different neural circuitries had been subject to investigation by different sub-disciplines of the neuroethological research (reviewed in Goodson & Kingsbury, 2013; McCall & Singer, 2012; O'Connell & Hofmann, 2011, 2012; Weitekamp & Hofmann, 2014).

Even though studies on hormonal regulation of sexual behavior and characteristics dates back to Arnold Berthold's experiments in the 1800s, behavioral neuroendocrinology, as we know it, began with the works of Frank A. Beach, William C. Young, and Daniel Lehrman during 40s and 50s. The primary focus this research agenda was to elucidate the neural correlates of hormone actions, thus among the main targets were the brain regions that possess steroid

receptors. Traditionally, mainstream behavioral neuroendocrinology was a subfield of physiological psychology and was critical of anthropocentrism and inclined to comparative approaches (Beach 1950; Hodos & Campbell, 1969) . Over last several decades, this research agenda was able to elucidate major proximate mechanisms of hormone actions in a variety of structures in the brain in association with adaptively significant behaviors. Drawing on these empirical and theoretical implications, Sarah W. Newman (1999), proposed an evolutionarily conserved brain circuitry, "social behavior network" which is specifically implicated in the control of a variety of adaptive social behaviors. This network consisted of the amygdala, bed nucleus of stria terminalis, lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal gray. All of these structures contain sex steroid receptors thus responsive to hormonal state of the animal, are bi-directionally connected to one other, and involved in adaptively significant social behaviors such as aggression, sexual behaviors, bonding and parental behavior (reviewed in Goodson, 2005). Newman (1999) argued that each adaptively significant stimulus was associated with a distinct pattern of activity in these afore mentioned structures (See figure 2).

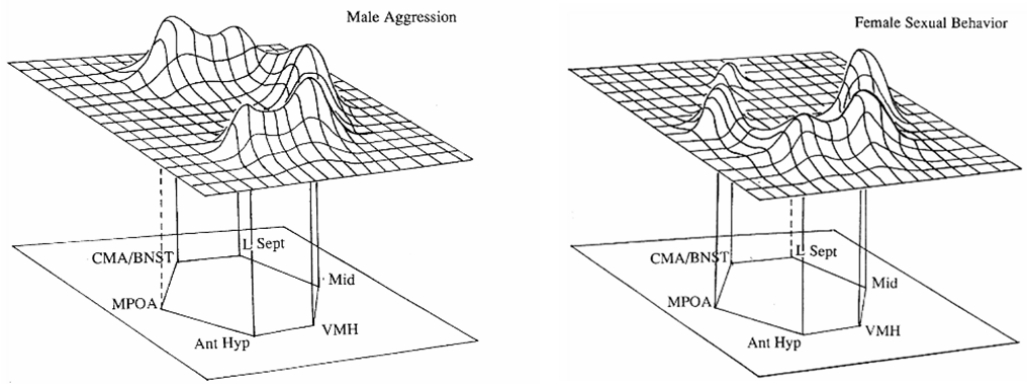

Figure 2. Hypothetical representations of the patterns of activity in the social behavior network in response different social behaviors. (Reprinted from Newman 1999, p250). According to Newman (1999), social behavior network consist of 6 nodes medial preoptic area (mPOA), ventromedial hypothalamus (VMH), lateral septum (L Sept), medial amygdala and bed nucleus of stria terminalis (CMA/BNST), midbrain periaqueductal gray (Mid)

The involvement of the structures of social behavior network in different social behaviors are well established, however, to this date no one has tested if these social behaviors are associated directly to distinct patterns of activation of the whole network. Nevertheless, the Social Behavior Network remains a useful framework to study adaptive social behaviors.

As discussed earlier in this chapter, there is also substantial amount of data relating mesolimbic system to naturally rewarding adaptive social behaviors. Based on this premise, O'Connell & Hofmann (2011) argued that these two systems, mesolimbic system and social behavior network, should interact with each in order for animals to produce flexible decisions in response social and environmental cues. And they've proposed a larger network; social decision-making network (SDM) which consists of the combination of structures in the social behavior network and the mesolimbic system in control of social behaviors in all vertebrates. This view has been criticized, in bases of vague homologies of mesolimbic structures, and lack of functional studies in a number of taxa (Goodson & Kingsbury, 2013). Goodson & Kingsbury (2013) contested that the "*SDM network model is most appropriately viewed as an important framework for generating hypotheses, rather than viewing SDM as a validated pan-vertebrate construct.*"

In a larger context, the data presented in this dissertation is interconnected with these afore mentioned neural network proposals, however, the primary aim is not to validate or reconcile these arguments. They are considered as a good theoretical framework, however his dissertation aims to elucidate interactions between POM and mesolimbic system and the specific empirical findings led us to explore this interaction is discussed in the following section.

V. Specific Aims

Early evidence from lesion studies implied that mPOA is a critical structure for consummatory sexual behaviors. Based on these findings Everitt (1990) argued that mechanisms controlling sexual arousal and copulatory were separate: mPOA regulating the consummatory sexual behaviors whereas ventral striatal system regulating sexual motivation. However, since then, there is accumulating evidence indicating that POM also plays a role in appetitive sexual behaviors, as previously discussed in microdialysis (Kleitz-Nelson et al., 2010) and immediate early gene (Taziaux et al., 2006) studies. In addition, in rats, projections from mPOA to VTA have been documented via tract tracing studies (Simerly & Swanson, 1988). Although limited, there are some studies indicating a functional significance to mPOA-VTA projections in relation to other motivated behaviors. For instance, in postpartum rats with unilateral lesions of mPOA, exposure to pups induced enhancement of IEG-ir in contralateral nucleus accumbens but not on the ipsilateral one where mPOA-VTA projections are disrupted (Stack, Balakrishnan, & Numan, 2002). Interestingly, mPOA lesions increased cocaine induced IEG-ir in nucleus accumbens and conditioned place preference in rats (Tobiansky, Roma, Hattori, & Will, 2013). Overall, these data indicate that projections from POM to VTA may have a modulatory role on different motivated behaviors.

Based on this empirical evidence we set out to elucidate if POM-mesolimbic system interactions are involved in the motivational aspects of sexual behavior, and if it is specifically through functional connectivity between POM and VTA.

In order to establish POM-mesolimbic system interactions we aimed to demonstrate that (1) the medial preoptic nucleus and (2) mesolimbic reward circuitry are associated with appetitive and consummatory aspects of sexual behaviors in male Japanese quail. In addition, (3) we need to determine the existence of afferent inputs originating in medial preoptic nucleus to

ventral tegmental area or mediating nuclei that enables this relationship and lastly (4) we need to demonstrate if these projections have a functional significance in relation to male sexual behaviors.

To address these questions, in **Chapter 2,** we aimed to demonstrate that different catecholaminergic and indolominergic cells groups of the social behavior network and mesolimbic reward circuit are associated with different aspects of male and female sexual behaviors by collecting brain tissue from quail that had engaged in sexual behaviors. Double-label immunocytochemistry procedure in which we co-localized the immediate early gene (IEG) Fos, with either tyrosine hydroxylase or tryptophan hydroxylase enabled us to investigate the specific nuclei implicated in different aspects of sexual behavior. Specially, this study provided the evidence that both POM and VTA were involved in both appetitive and consummatory aspects of sexual behavior. However, in this study were unable to quantify IEG-ir in nucleus accumbens, due to inconstancies of its anatomical localization in the literature. Therefore, in **Chapter 3**, we investigated both the homology and hodology of the avian nucleus accumbens. To establish this, we labeled tissue with immunohistochemical markers of the Serotonin Transporter (SERT), Dopamine-and cAMP-regulated phosphoprotein, Mr 32 kDa (Darpp-32), and Calretinin. Subsequently, in chapter 3 we used a retrograde tracing method and injected biotinlylated dextran amines (BDA) into Ac and investigate the efferent inputs to the Ac. Since the results of these anatomical studies allowed us to establish the boundaries of and hodology of the Ac, we then tested the functional homology of Ac via examining the early growth response protein (Egr-1) immunoreactivity in association to sexual behavior. The results demonstrate that the function of the avian Ac is also congruent with its mammalian homologue, and indicate a significant role in regulation of sexual rewards. **In Chapter 4**, based on the anatomical findings

from the previous chapter we did bilateral 6-hydroxydopamine (6-OHDA) lesions to deplete the dopamine within POM and Ac. Quail exposed to 6-OHDA exhibited a rapid impairment in both aspects of sexual behavior and this impairment persisted for 5 hr and 24hr after 6-OHDA injections in both POM and Ac compared to sham injections. However, there was complete recovery of these behaviors 1 week after surgery. Overall, this study demonstrates that dopaminergic innervation of the POM and Ac is necessary for the expression of appetitive and consummatory sexual behavior in male quail. In **Chapter 5,** POM and VTA were unilaterally inactivated, via muscimol, either ipsilaterally or contralaterally. We documented that when administered unilaterally muscimol has negligible effects on both appetitive and consummatory sexual behaviors whereas contralateral inactivation impaired the appetitive sexual behaviors but had no significant effects on consummatory sexual behaviors. This finding implied that POM-VTA connectivity might be associated with appetitive sexual behaviors. In **Chapter 6,** we stereotaxically injected a biotinylated dextran amine (BDA) into VTA as a retrograde neuroanatomical tracer. Our results demonstrated a significant increase in Fos-immunoreactivity (ir) among the retrogradely labeled BDA cells in POM and LHA for quail in sexual behavior condition, indicating a functional hodology between the nuclei.

Chapter 2 - Fos Expression in Monoaminergic Cell Groups in Response to Sociosexual Interactions in Male and Female Japanese Quail

Rationale

As was mentioned in the previous chapter, there is substantial evidence indicating that dopaminergic, noradrenergic and serotonergic cell groups are involved in the regulation of social interactions associated with sexual reproduction in a variety of vertebrate species (Balthazart & Ball, 1998; Pfaus, 2009). In particular, numerous studies have addressed the excitatory role of DA and inhibitory role of serotonin (5-HT) especially in the regulation of male sexual behavior (Hull, Muschamp, & Sato, 2004) . For example, in vivo microdialysis in the medial preoptic area (mPOA) demonstrated an increase in extracellular DA activity during precopulatory exposure to female conspecifics in male quail (Kleitz-Nelson, Dominguez, & Ball, 2010) and rats (Elaine M Hull, Muschamp, & Sato, 2004). Furthermore, D1 dopamine receptor agonists facilitated copulatory behaviors and prolonged the time spent with the estrus female in male rats (Beck, Biały, & Kostowski, 2002). In male quail, appetitive and consummatory behaviors decreased when animals were treated with D1 receptor antagonists, but increased when animals were treated with D1 receptor agonists (Balthazart, Castagna, & Ball, 1997). This line of research indicated the importance of DA both in initiation of sexual behavior and copulatory performance, and emphasized the role of dopamine on motivational aspects of the sexual behavior.

In contrast, serotonin is known to have an inhibitory effect on male and female sexual behavior. Studies in various vertebrate species indicate that endogenous serotonin release contributes to the onset of sexual satiety (Hull et al., 2004). For example, administration of 5-HT to mPOA impaired male sexual behavior in rats (Fernández-Guasti, Escalante, Ahlenius, Hillegaart, & Larsson, 1992). Additionally, in vivo microdialysis demonstrated that 5-HT levels

increased in the lateral hypothalamic area after ejaculation in rats but were stable during the presence of female and copulation (Lorrain, Matuszewich, Friedman, & Hull, 1997), which is in agreement with the notion that activation of the serotoninergic system is implicated in the development of sexual satiation. Pharmacological studies indicate that noradrenaline (NA) plays a similar role to 5-HT in male sexual behavior. A NA receptor antagonist, yohimbine, reversed the sexual inhibition due to sexual exhaustion (Rodríguez-Manzo & Fernández-Guasti, 1994). Also, injection of noradrenergic neurotoxin (DSP4) facilitated sexual behavior in male quail, indicating NA's inhibitory role in sexual behavior (Balthazart, Libioulle, & Sante, 1988).

Immediate early gene studies (IEG) have been very valuable in identifying brain areas that are involved in the various components of sexual behavior in quail (Charlier, Ball, & Balthazart, 2005; Meddle, Foidart, Wingfield, Ramenofskyand, & Balthazart, 1999; Taziaux et al., 2006). These studies demonstrated an increase in IEG expression in a number of nuclei in response to different components of sexual behavior and therefore established critical evidence for the parts of the neural circuitry controlling the different aspects of sexual behavior. In addition, studies that are investigating effects of testosterone on female appetitive and consummatory behavior indicated that different neuroendocine mechanisms are involved in different aspects of sexual behavior as evident from differentiated IEG expression in preoptic medial nucleus (POM) of female quail (Balthazart, de Meaultsart, Ball, & Cornil, 2013).

Even though, these pharmacological and IEG studies provided valuable evidence concerning the roles of different monoaminergic neurotransmitters in modulation of sexual behavior, the specific role of different monoaminergic cell groups is still not well understood. In particular these studies did not investigate the roles of: 1) indolaminergic cell groups in different aspects of sexual behavior, 2) catecholaminergic cell groups in relation to appetitive sexual

behavior in males 3) either of these cell groups in naturally occurring female appetitive or consummatory behaviors. Therefore, the goal of this chapter was to examine the roles of these monoaminergic cell groups in different aspects of sexual behavior in male and female quail.

One particular way of investigating the effects of specific monoaminergic cell groups on sexual behavior is combining expression of immediate early genes (IEGs), like Fos, with tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) immunohistochemistry. TH is the rate-limiting enzyme for catecholamine biosynthesis and TPH plays the same role in the synthesis of indolamine. Hence, we mapped here the induction of the c-fos protein via immunohistochemistry in two different sets of sections that were the double-labeled for TH or TPH in brains of quail of both sexes collected after they expressed both aspects of sexual behavior.

Materials and Methods

Subjects

A total of 48 experimentally naïve adult (10 weeks old) male (24) and female (24) Japanese quail (*Coturnix japonica*) were obtained from a local breeder. All subjects were maintained on a standard 16L/8D cycle at approximately 22°C and had food and water available *ad libitum*. Both male and female quail were housed in individual cages throughout the experiment. All of the experimental procedures were in accordance with Johns Hopkins University Animal Care and Use Committee guidelines.

Apparatus

Behavioral testing took place in an aquarium that consisted of two compartments separated by opaque and transparent sliding panels. A camcorder was placed under the aquarium to allow recordings of the cloacal area to allow assessment of rhythmic cloacal sphincter movements (RCSMs), a measure of appetitive sexual behavior (see below) and another one placed in front of aquarium to provide measures of the consummatory sexual behavior, namely the frequency of neck-grabs (NG), mount attempts (MA), mounts (M) and full cloacal contact movements (CCM).

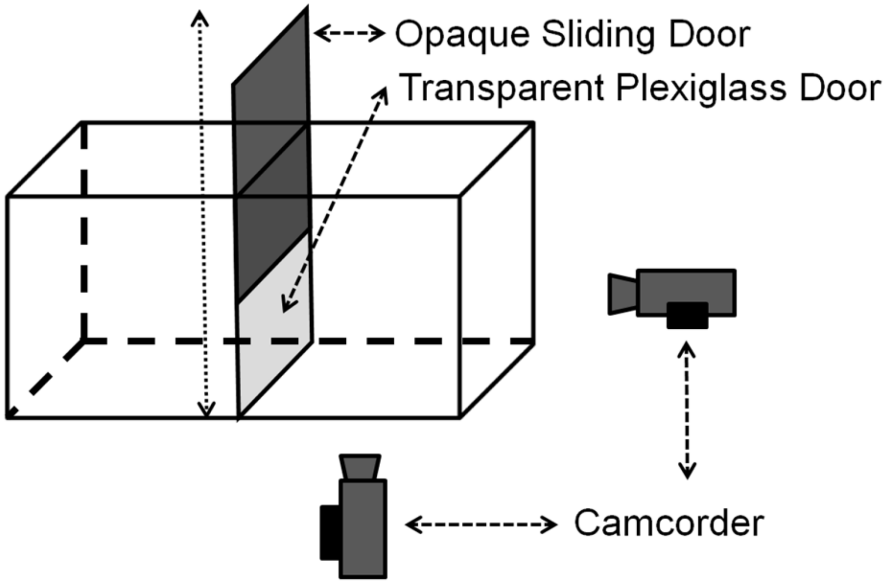

Figure 3. A schematic representation of testing apparatus.

Experimental Procedures

Quail were randomly assigned to three groups in consistent pairs of one male and one female: visual contact (VC), physical contact (PC), and Control groups (n=8 per group) that remained the same throughout the experiment. At the start, all subjects experienced five consecutive days of a 15 minute pretest in which males and females interacted freely and gained

sexual experience. Before the first pretest, baseline RCSM frequencies of all males were collected during 5 minutes without the presentation of female. In addition, animals were individually placed daily in a holding cage (HC) for 15 minutes for nine successive days to habituate them to the environment and handling. A five day break was given between pretests and the final testing while subjects continued to be placed daily in the HC (see figure 4).

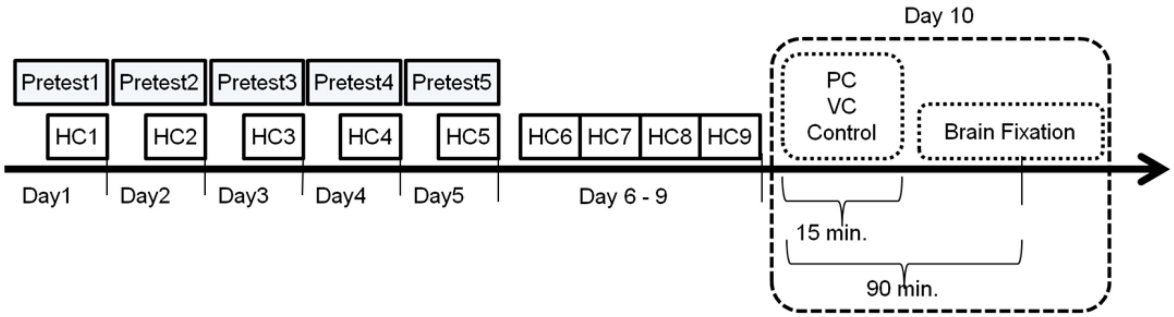

Figure 4. A schematic representation of experimental time line indicating days of pretesting for gaining sexual experience, holding cage (HC) placements for environmental habituation and experimental manipulations.

The final testing took place on the 10th day. Like during the pretests, one male and one female quail were placed in the two different sides of the experimental chamber. In the VC group, first, RCSM frequencies of males were measured for 5 minutes, and then the opaque panel was removed for 15 min while the Plexiglas panel remained in place allowing only visual access to the bird of the opposite sex. Once again, the RCSM frequencies of all male subjects were measured for 5 minutes starting with the onset of visual access to the female because previous work showed that RCSM frequencies rapidly decrease in these conditions (de Bournonville et al., 2013). For females, RCSM measurements were not collected, because these contractions are not produced in the presence of male quail as a correlate of appetitive behavior under natural conditions (Adkins-Regan & Leung, 2006).

For the PC group both panels were removed and the male and female quail freely interacted for 15 minutes. In this group subjects thus experienced bodily contact with the partner of the opposite sex associated with somatosensory stimulation as well as visual, auditory and possibly olfactory interactions. During the first 5 minutes of this period, frequencies and latencies of the first occurrences of NG, MA, M and CCM were recorded. These behavioral frequencies also markedly decline after the first few minutes of interaction (De Bournonville et al., 2013). Animals in the control group were placed in the holding cage they had been habituated to for 15 minutes. Animals from all three groups returned to their home cages after the 15 minute manipulation and remained in the home cages for the next 75 minutes until their brains were collected.

Fixation and Immunohistochemistry

Ninety minutes following onset of the behavioral tests, subjects were decapitated and their brain dissected out of the skull. The brains were placed into acrolein (5% in phosphate buffer 0.1 M saline) for 3 hours, washed four times in PBS (15 min) and cryoprotected in 30% sucrose for 24 h at 4 °C. The brains were then frozen on dry ice and stored at −70 °C until used. All brains were cut at 35 μm in the coronal plane using a cryostat at –20°C and sections were collected in four series.

Fos expression was then visualized by immunohistochemical procedures with the Avidin Biotin Complex (ABC) technique. Three rinses with 0.01 M PBS containing 0.1% Triton X-100 were performed between each step. First, sections were incubated for 60 min in 0.3% hydrogen peroxide and in 20% normal goat serum to remove endogenous peroxidase and decrease non-specific binding. This step was followed by Avidin-biotin blocking (Vector SP-2001) for 15 min,

to block possible biotin binding sites in the tissue. Then sections were incubated in the primary Fos antibody (1:10,000) for 48 hours. Afterwards, sections were incubated for 60 min in goat anti-rabbit serum. The antibody-antigen complex was localized using the avidin-biotin complex method performed with a Vector Elite Kit (ABC Vectastain Elite PK-6100, Vector Laboratories PLC) and finally, the peroxidase enzymatic activity was visualized with DAB (3,3'diaminobenzidine tetrahydrochloride) intensified with Nickel ammonium sulfate and chloride.

A similar technique was used for immunohistochemical labeling of TH and TPH in two different series of sections that were already labeled for Fos with the following exceptions. Sections were incubated in anti-TH antibody (1:10,000, Immunostar AB22941) or anti-TPH antibody (1:10,000, Millipore AB938) for 48 hours. Afterwards, sections were incubated for 60 min in biotinylated horse anti-mouse IgL for TH and biotinylated donkey anti-sheep IgL for TPH. The peroxidase enzymatic activity was visualized with DAB alone. Reactions were terminated by several rinses in PBS and sections were mounted and coverslipped.

Quantification of Immunohistochemical Results

Cells immunoreactive (ir) for Fos and cells double-labeled by Fos and either TH or TPH were quantified in several brain nuclei selected based on previous work either identifying Fos induction by sexual behavior or presence of catecholaminergic or indolaminergic cells groups or both (Ball & Balthazart, 2004; Charlier, Ball & Balthazart, 2005; Meddle et al., 1997; Meneghelli et al., 2009; Tlemçani et al., 2000; Taziaux et al., 2006). Quantification of all Fos-ir, Fos-ir+TH-ir & Fos-ir+TPH-ir cells was done by an experimentally blind observer under a light microscope by direct observation (see figure 5 & 6). To validate these counts a different

experimentally blind observer collected data for POM, BSTM, ventral tegmental area (VTA, A10 dopaminergic cells), periaqueductal gray (PAG, A11 dopaminergic cells) and raphe pallidus (Rp) and a high correlation between the observers was present (r (186) = 0.923, p<.001).

Preoptic Medial Nucleus (POM)

Fos-ir nuclei were quantified in a rectangular field of 0.58 mm^2 aligned with the ventral edge of the anterior commissure and the lateral edge of the third ventricle at a rostro-caudal level including the largest extension of the anterior commissure that corresponds to plate 14 (Interaural 3.76 mm) of the chicken atlas of Puelles, Martinez-de-la-Torre, Paxinos, Watson, & Martinez, (2007) (see figure 5 A & B).

Bed Nucleus of Stria Terminalis (BSTM)

Fos-ir cells were quantified in the BSTM at a slightly more caudal level corresponding to plate 15 (Interaural 3.28 mm) where the anterior commissure has just disappeared and the BSTM has adopted a recognizable V-shape. Quantification was performed in two rectangular 0.42 mm^2 fields, one adjacent to the third ventricle and one moved one full field more dorsally and half a field laterally to capture most of the "V" shape of the BSTM (Figure 5 C-F).

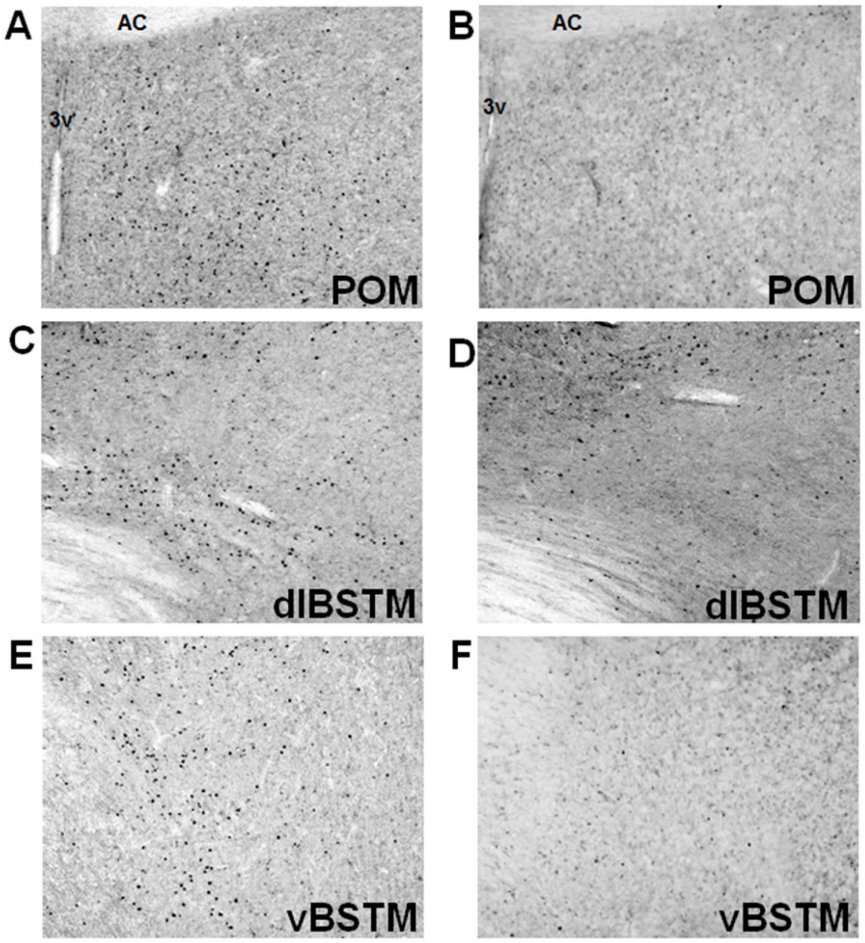

Figure 5. Photomicrographs illustrating the brain regions where Fos-immunoreactive (ir) cells were quantified. Panels A-B illustrate the Fos-ir cells at the rostro-caudal level of the medial preoptic nucleus (POM) located just ventral to the anterior commissure (AC) and lateral to the third ventricle (3v) for males in the physical contact (A) and control (B) groups. Sections C-F illustrate Fos-ir cells in the dorsolateral (C-D) and ventral (E-F) bed nucleus of stria terminalis (dlBSTM and vBSTM) respectively for males in the physical contact (C and E) and control (D and F) groups.

Catecholaminergic Areas

Catecholaminergic areas (A10 dopaminergic cells of ventral tegmental area, VTA; locus coeruleus, LoC; A9 dopaminergic cells of substantia nigra SN; subceruleus ventrale, SCv; A11 dopaminergic cells of anterior (a) and posterior (p) periaqueductal gray, PAG) were localized based on the chicken atlas of Puelles et al., (2007) and (Charlier, Ball & Balthazart, 2005). The borders of these nuclei were identified by the high density TH labeling and/or surrounding

anatomical markers (Figure 6 B-D). Single FOS-ir, and double FOS-ir/TH-ir cells were counted under a light microscope (40X objective) by direct observation (see figure 6 A).

Serotonergic Raphe Pallidus Nucleus

The Raphe Pallidus Nucleus (Rp) is located ventral to the medial longitudinal fasciculus in plate 49 (Interaural -4.88mm) of the chicken atlas of Puelles et al., (2007) and can be defined by the high density of TPH positive cells (see Figure 6 F). Single Fos-ir and double Fos-ir/PTH-ir cells were counted under a light microscope (40X objective) by direct observation in this nucleus (see Figure 6 E).

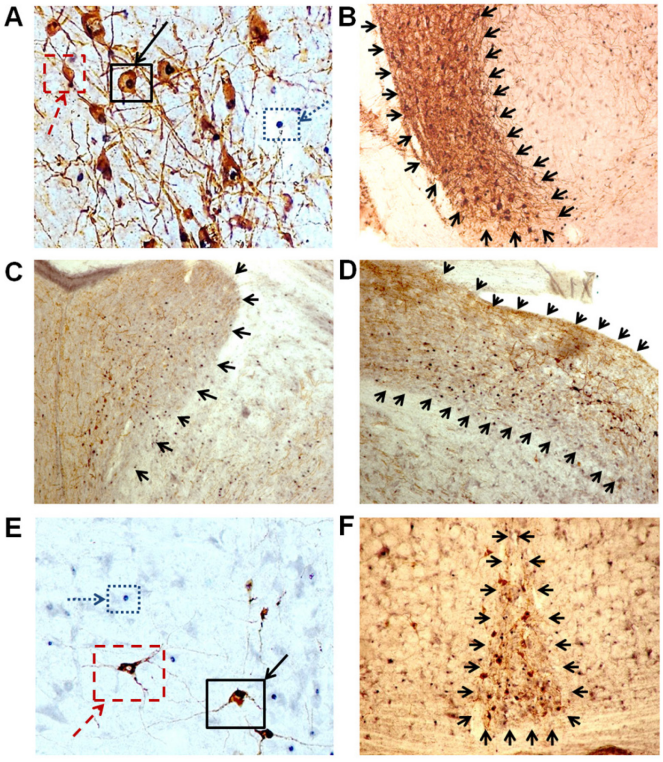

Figure 6. Photomicrographs illustrating the Fos-ir+TH-ir (A-D) and Fos-ir+TPH-ir (E-F) double labeled cells within the regions of interest. Panel A illustrates a Fos-ir positive (blue arrow), TH-ir positive (red arrow) and doubled labeled cell (black arrow) in the substantia nigra (A9). Panels B-D illustrates Fos and TH positive cells in the ventral tegmental area (A10) (B), anterior periaqueductal gray (A11) (C), and posterior periaqueductal gray (A11) (D), blacks arrows demarcate the borders of these nuclei. Panel E illustrates a Fos-ir positive (blue arrow), TPH-ir

positive (red arrow) and doubled labeled cell (black arrow) in the raphe pallidus nucleus. Panel F illustrates the Fos-ir and TPH-ir positive cells in raphe pallidus nucleus, blacks arrows demarcate the borders of the nucleus.

Results

Behavioral Data

In the VC male group, the RCSM frequencies were collected in three different 5 minute time periods. The first one was on the first testing day to assess the baseline RCSMs, prior to any sexual experience. The second one was on the last day of testing prior to visual access to female and the third one was on the onset of visual presentation of female. A repeated measures one-way ANOVA indicated a significant difference ($F(2, 14) = 69.16$, $p<.05$, corrected for non-sphericity with Greenhouse-Geisser) between these three conditions. The contrast analysis between baseline RCSM vs. pre-visual RCSM and pre-visual RCSM vs visual RCSM exceeded the critical F value depicted by Scheffe's S test ($Fs=(2)(2,14)=7.4$) which indicated a significant difference between them (see figure 7 A).

In the PC male group, the mean latency of sexual approach was 156.6 sec. on the first day of testing whereas it was 2.75 sec on the 10^{th} day just before brain collection. Paired sample t test demonstrated a significant decline in latency of sexual approach ($t(7)= 3.390$, $p < .05$; Figure 7 B). A significant increase of the frequency of neck-grabs ($t(7)= 3.389$, $p <.05$), mount attempts ($t(7)= 2.434$, $p <.05$), mounts ($t(7)= 2.538$, $p <.05$) and cloacal contact movements ($t(7)= 4.432$, $p <.05$) was also observed between these two tests performed on day 1 and 10 (see Figure 7 C).

These behaviors are not displayed by females during their physical interaction with males and the corresponding data are thus not available.

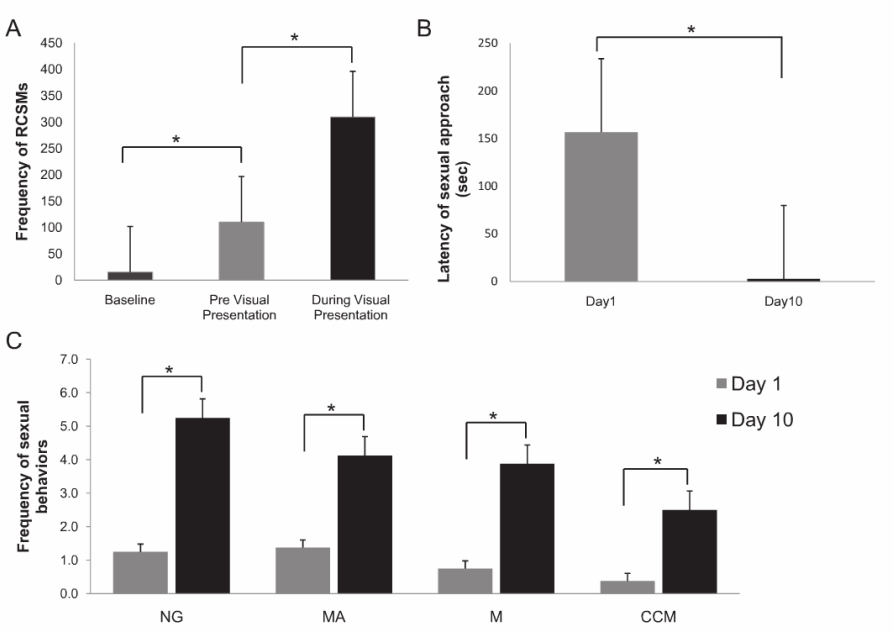

Figure 7. (A) Mean (± 1 SE) frequency of RSCMs within 5 min quantification periods for baseline, prior to visual access and during visual access. (B) Mean (± 1SE) latency of first sexual approach within 5 min quantification periods. (C) Mean (± 1SE) Frequency of neck-grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM).

Fos Expression in POM & BSTM

An analysis of variance (ANOVA) comparing the mean number of Fos-ir cells in POM among different experimental conditions showed a significant difference between male groups ($F(2.21)=3.972$, $p<.05$) but not in females ($F(2, 20)=1.042$, $p>.05$). Post hoc analysis by the Fisher's least significant difference (LSD) test indicated an increased number of Fos-ir nuclei in POM in the PC ($p<.05$) and VC ($p<.05$) groups when compared to control condition in male quail (Figure 8 A-B).

Similarly, ANOVAs indicated a significant difference among groups ($F(2, 18) = 4.023$, $p<.05$) in the mean number of Fos-ir in the BSTM of male quail. Post hoc analysis (LSD) demonstrated a significant increase in Fos immunoreactivity in PC ($p<.05$) and VC ($p<.05$)

groups when compared to control. No significant difference was found between female groups, ($F(2, 21)=.735$, $p > .05$) (Figure 8 C-D).

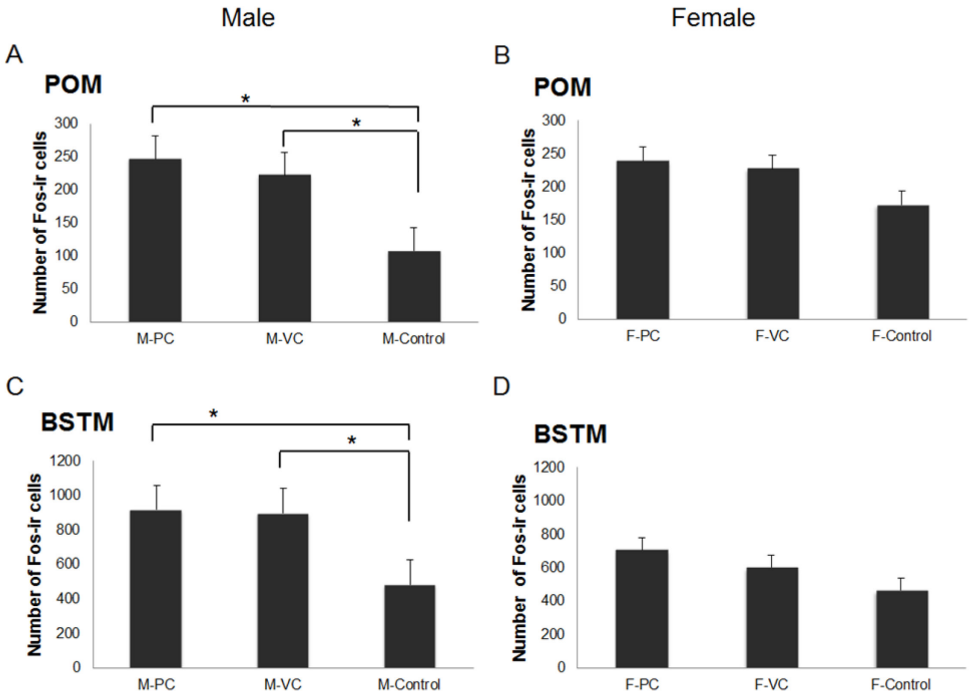

Figure 8. Mean (±1 SE) number of Fos-ir cells in the POM (A-B) and BSTM (C-D) of male (left) and female (right) quail. Asterisks indicate significant differences by post hoc LSD analysis.

Fos Expression in VTA

Figure 9 presents the mean number of Fos-ir and TH+Fos-ir double labeled cells within VTA. Significant group differences were found in male quail for Fos-ir cell numbers ($F(2.19)=4.124$, $p < .05$) but not for TH+Fos double labeled cells ($F(2.19)=2.037$, $p > .05$). Post hoc analysis by the LSD test showed a significant difference between the PC and Control ($p < .05$) and between VC and Control ($p < .05$) groups (see figure 9 A-B).

In female quail, ANOVA also indicated a significant difference in number of Fos-ir cells between the three experimental groups ($F(2, 22)=16.337$, $p < .05$). Post hoc analysis (LSD)

indicated a significant difference between PC and Control (p <.05) and between PC and VC (p <.05) groups. No significant difference between groups was observed for the double labeled cells in the female VTA (F(2.20)=0.797, p >.05; see figure 9 C-D).

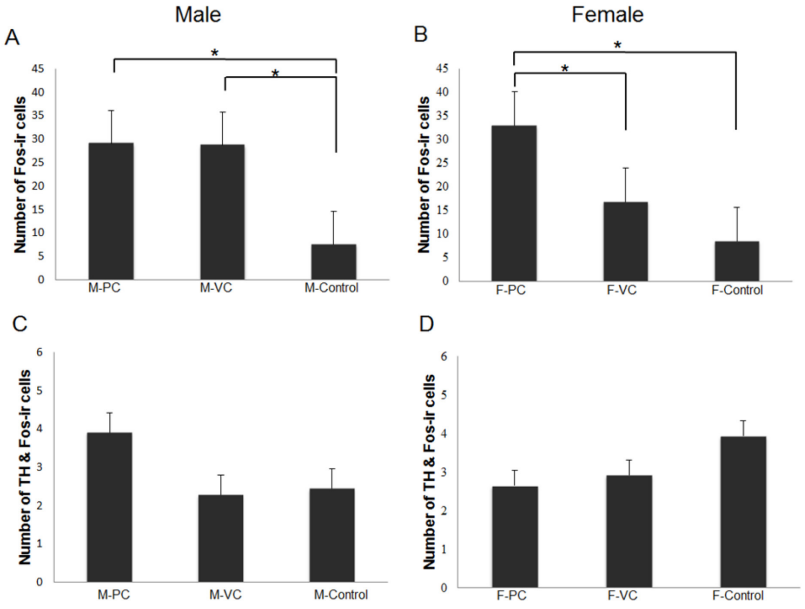

Figure 9. Mean (±1 SE) number of cells in the VTA (A10) of males (left) and females (right) that were labeled for Fos only (A and B respectively) or were double labeled for TH and Fos (C and D respectively). Asterisks indicate significant differences identified by post hoc LSD analysis.

Two of the analyses reported above that concern male and female Fos-ir in VTA did not meet the homoscedasticity assumption. Therefore, to avoid drawing erroneous conclusions related to a possible violation of the ANOVAs conditions, we additionally ran non parametric Kruskal-Wallis tests for all these data. The statistical outcomes were the same as reported above for parametrical analyses, which confirms the conclusions reported in this paper.

Fos Expression in PAG

In the anterior PAG, a significant group difference was present for Fos-ir cells of male quail (F(2, 19)=5.365, p <.05). The post hoc analysis indicated a significant difference between the PC (p <.05) and VC (p <.05) and the control condition. No significant group difference was

present for the double labeled cells within the anterior PAG ($F(2,19)=2.296$, $p > .05$) (see figure 10 A & C).

In females a significant difference among groups was also present in the anterior PAG, for single ($F(2, 17)=6.130$, $p < .05$) and also for double labeled cells ($F(2, 17)=5.114$, $p < .05$). Post hoc analysis (LSD) demonstrated that female quail in the PC group had elevated number of Fos-ir ($p < .05$) and of TH+Fos-ir ($p < .05$) cells compared to the control and VC groups respectively (see figure 10 B & D).

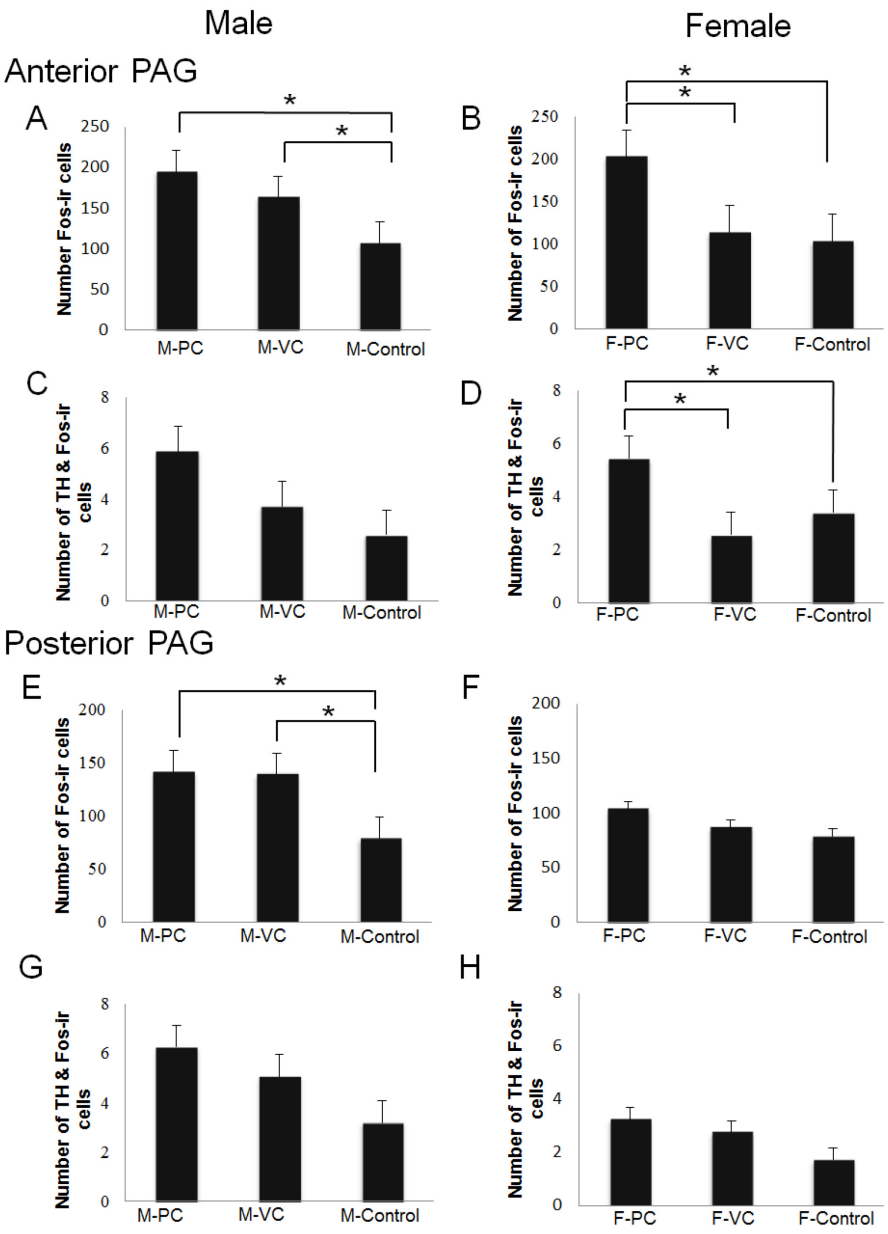

Figure 10. Mean (±1 SE) number of Fos-ir (A-B, E-F) or TH+Fos-ir (C-D, G-G) cells in males (left) and females (right) in posterior and anterior PAG (A11). Asterisks indicate significant differences as identified by post hoc LSD analysis.

In the posterior PAG a single significant group difference was found: numbers of Fos-ir cells differed between groups in male quail ($F(2, 18) = 6.212$, $p < .05$) but not in female quail ($F(2, 18) = 1.205$, $p > .05$). In males, post hoc analysis (LSD) yielded a significance difference

between PC and control (p <.05) and between VC and control (p <.05) groups for single Fos-ir cells. No significant difference was present in numbers of double labeled cells in male (F (2, 17)=1.420, p >.05) nor female quail (F (2, 21)=2.267, p >.05) (see figure 10 E-H).

IEG Expression in SN

ANOVAs comparing the mean number of Fos-ir cells in SN among different experimental conditions showed no significant difference for males (F (2, 16)=1.743, p >.05) nor females (F (2, 19)=1.351, p >.05). In addition, no significant difference was found for TH-Fos double labeled cells in males (F (2, 16)=0.440, p >.05) and females (F (2, 19)=1.619, p >.05).

Fos Expression in Noradrenergic Cell Groups: LoC and SCv

No significant group difference in the number of Fos-ir cells, in males (F (2,18)=2.287, p >.05) and females, (F (2,21)=.876, p >.05), or in double labeled cells in males, (F (2,18)=2.220, p >.05) and females, (F (2,21)=1.964, p >.05) were observed.

Similarly, in SCv, ANOVA also did not indicate any significant group differences in single Fos-ir cells for males (F (2, 18)=1.238, p >.05) and females (F (2, 19)=1.407, p >.05) or in double labeled cells for males (F (2, 18)=1.088., p >.05) and females (F (2, 19)=0.730, p >.05).

Fos Expression in Serotonergic Raphe Pallidus Nucleus (Rp)

The ANOVAs comparing the number of single labeled Fos-ir cells in the three groups of males indicated a significant difference (F(2, 19)=5.323, p <.05; figure 11 A). In addition post hoc LSD tests indicated a significant difference between PC - VC (p <.05) and PC - Control (p

<*.05*). The corresponding analysis found no significant difference in the number of Fos-ir cells between the three groups of females (F(2, 19)=.182, *p* >.05; figure 11 B).

The ANOVA indicated a significant group difference in the numbers of TPH+Fos immunopositive cells within the raphe pallidus nucleus of male quail (F(2, 19)=9.11, *p* <.05). Post hoc analysis by LSD test yielded a significant difference between the PC and VC (*p* <.05) and the PC and Control (*p* <.05) groups (see figure 11 C). No significant group difference in the numbers of double-labeled cells was found in female quail (F (2, 19)=0.66, *p* >.05; figure 11 D).

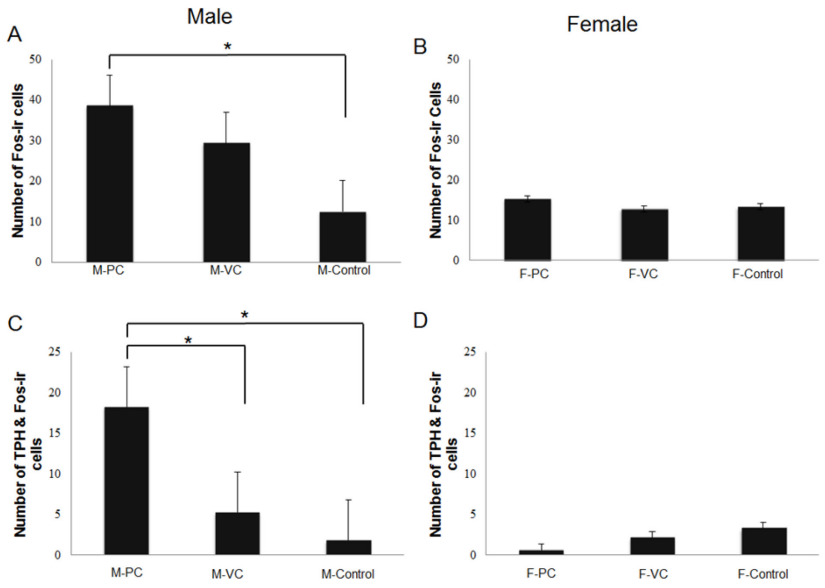

Figure 11. Mean (±1 SE) number of Fos-ir (A-B) and of TPH+Fos-ir (C-D) cells in the raphe pallidus nucleus of males (left) and females (right). Asterisks indicate significant differences by post hoc LSD analysis.

Discussion

The present chapter revealed an enhanced Fos immunoreactivity in a variety of brain nuclei subsequent to expression of appetitive sexual behaviors in male quail and of consummatory sexual behaviors in male and female quail. In POM and BSTM, this increase was present after expression of both aspects of sexual behavior but only in males. Fos induction

associated with consummatory behavior was also observed in different dopaminergic nuclei— the VTA and the anterior PAG— in both sexes but a similar increase in Fos-ir cells number was observed only in males in the appetitive (visual contact) condition. In the posterior PAG, the increase of Fos expression was associated with both aspects of sexual behavior and was observed exclusively in males. In noradrenergic cell groups, the LoC and SCv, we observed no significant difference after expression of both components of sexual behavior. In addition, we observed that consummatory, but not appetitive, sexual behavior induced Fos expression in the serotonergic nucleus of raphe pallidus in male but not in female quail. Overall, the present study demonstrates that different monoaminergic cell groups are specifically activated, in a sex specific manner, in relation to different aspects of sexual behavior.

Behavioral Results

In concordance with previous studies on appetitive sexual behavior (Seiwert & Adkins-Regan, 1998) , a clear increase of Rhythmic Cloacal Sphincter Movements (RCSMs) frequency was present in male quail visually exposed to a female. In addition, just placing the male in the experimental chamber where copulation had occurred during previous tests significantly increased the RCSM frequency without any visual access to a female although to a lower level, suggesting the presence of a form of conditioning such that the anticipation of a female and of the possibility to express sexual behavior became sufficient to activate this behavior. A similar form of conditioning of RCSMs has been previously reported in experiments that associated the repeated presentation of a neutral stimulus with the view of a female (Cornil et al.,2004; Holloway, Balthazart, & Cornil, 2005). It should be noted that this behavioral conditioning, if it potentially influenced the frequency of RCSM on the final test day, cannot be invoked to explain

the group differences in Fos induction observed after the final behavioral test. All birds in the three different groups had indeed been exposed to the same pretests and had spent similar amounts of time in the holding cage. This identical past experience thus cannot be the source of any differential pattern of Fos induction.

A pronounced increase of RCSM frequency was also observed when we compared the pre-visual with the visual condition in male quail which is indicative of an enhancement of the appetitive behaviors in these males. Under physiological endocrine conditions, i.e., in the absence of treatment with exogenous testosterone, female quail do not show male-typical RCSMs when provided with visual access to a male quail (Adkins-Regan & Leung, 2006; Balthazart et al., 2013). Their female-typical appetitive responses are also very discrete and irregularly observed when birds are tested in a confined enclosure so that they are difficult if not impossible to quantify. Therefore, although no behavioral measurements were collected for female in quail in the appetitive condition, one of the aims of the present study was to investigate the neural correlates of the visual exposure to a male in order to obtain in both sexes estimates of the neural responses to the same conditions i.e. visual or physical exposure to a sexual partner. This investigation allowed us to evaluate possible sex differences in this aspect of sexual behavior.

IEG Results

Findings of the present study strengthen the view that POM has a critical role in male sexual behavior. Converging evidence from different lines of research including other IEG studies (Charlier, Ball & Balthazart, 2005; Meddle et al., 1997 ; Taziaux et al., 2006), effects of electrolytic lesions (Balthazart & Surlemont, 1990a) , stereotaxic injections of aromatase

inhibitors (Balthazart, & Surlemont, 1990b) and testosterone implants (Bailhache et al., 1993; Balthazart et al., 1990) all support this conclusion.

The current study did not find any difference in the expression of Fos-ir material in POM between different groups of females. Previous IEG studies have also failed to find a significant effect of sexual interactions on Fos expression in the POM of female quail exposed to their endogenous gonadal secretions (Balthazart et al., 2013; Meddle et al., 1999). Anatomical and hodological studies also imply that POM is more critical for regulation of sexual behavior in males. For instance, the volume of the POM is sexually dimorphic and smaller in females than in males (Panzica et al., 1987) . Moreover, female quail have fewer aromatase-positive neurons projecting to the PAG than males (Carere et al., 2007) .

We also found an increased number of Fos-ir cells in the BSTM of male, but not female, quail subsequent to both appetitive and consummatory sexual behavior conditions. Electrolytical lesions to POM and BSTM were previously shown to differentially influence male appetitive and consummatory behaviors: lesions to BSTM had no effect on another measure of the male appetitive sexual behavior, the learned social proximity response but they did have an inhibitory effect on consummatory sexual behaviors (Balthazart et al. 1998). However, findings of the present study indicate that both appetitive and consummatory sexual behaviors increase the number of Fos-ir nuclei in POM and BSTM for males suggesting that both structures are implicated in both component of sexual behavior.

IEG Induction in Dopaminergic Cell Groups

Male quail that expressed the consummatory sexual response showed increased Fos expression in the VTA, as reported in a previous study (Charlier, Ball & Balthazart, 2005). The

present study also revealed a consummatory behavior-induced Fos expression in the VTA of females. However, we observed an increase in Fos-ir following appetitive sexual behavior in males but not in females.

We failed to identify a significant increase in TH-Fos double labeled cells in VTA and PAG in association with sexual behavior. Similar to our findings, initial analysis of the Charlier et al., (2005) study, also failed to demonstrate changes of TH-Fos-ir cell numbers in the VTA and PAG. However further analysis in the aforementioned study, which combined different experimental groups engaged in sexual behavior or controls, demonstrated a significant difference in TH-Fos-ir expression. Unlike in the Charlier et al. (2005) study, the design of the present study was not suitable for combining groups to increase the sample size. Thus our sample size was possibly insufficient to provide the statistical power needed to detect the changes in Fos expression specifically in TH-positive cells in VTA and PAG.

Previous work employing IEG expression has also identified changes in gene expression specific to midbrain dopaminergic cells in association with sexual behavior. For instance in zebra finches courting and mounting behaviors were associated with an increase in Fos expression in dopaminergic cells of VTA and PAG (Bharati & Goodson, 2006). The same study, also demonstrated a correlation between directed songs, as an indicator of appetitive sexual behavior, and TH-Fos-ir cell numbers in the PAG. This activation of midbrain dopaminergic neurons in association with sociosexual interactions has also been reported in mammals (i.e. Balfour, Yu & Coolen, 2004). Other studies also noted a positive correlation between TH density and directed songs and courtship behaviors in VTA (Alger, Juang, & Riters, 2011). Overall, these studies strongly suggest that dopaminergic neurons within VTA and PAG are associated with sexual

behaviors and the effect observed here on the total number of Fos-ir cells in VTA and PAG is also in concordance with these findings."

It could in addition or alternatively be considered that the neuronal activation observed in the VTA and PAG of sexually active males concerns mostly the non-dopaminergic neurons (e.g. GABAergic cells; see Tolu et al., 2013) and that these neurons indirectly activate dopaminergic cells, either after a longer latency or through another metabolic route that does not involve Fos expression.

Hodological studies of POM and VTA have demonstrated that there are ascending dopaminergic projections originating from VTA and incertohypothalamic DA cells to POM (Balthazart & Absil, 1997). Also, in vivo microdialysis studies demonstrated a release of dopamine in the male mPOA during performance of appetitive and consummatory sexual behavior (Kleitz-Nelson et al., 2010) and during copulation in rodents (Hull et al., 1995). Studies in rodents also demonstrated that incertohypothalamic DA projections to mPOA are associated with sexual behavior (Bitran et al., 1987).

It is important to note that VTA also plays a critical role in the mesolimbic reward system via its projections to nucleus accumbens, and in mammals a number of studies showed that this circuitry is involved in sexual behavior (reviewed in Frohmader, et al., 2010; Pfaus, 2009). There are a number of studies indicating a similar role of VTA in the mesolimbic reward system in birds. For example, a positive correlation has been noted between directed songs and courtship behaviors and the density of TH-ir cells in the VTA and nucleus accumbens in zebra finches (Alger, Juang, & Riters, 2011). Also, an increase was observed in the number of IEG-ir cells in the VTA and nucleus accumbens of female white-throated sparrows in response to hearing

conspecific song (Earp & Maney, 2012). Thus, the observed increase of the Fos-ir in VTA in association with sexual behavior could be related to activation of the mesolimbic reward system.

Induction of Fos in PAG was different in males and females and specialized in different subdivisions of the nucleus. There was no significant Fos induction in the posterior PAG of females whereas increased Fos-ir was present after expression of PC in the anterior PAG of females. In males, elevated Fos expression was present after performance of VC and PC throughout the rostro-caudal extent of PAG. Previous studies demonstrated sexual behavior-induced Fos immunoreactivity within PAG in males (Charlier, Ball & Balthazart, 2005) and our study now adds consummatory behavior-induced Fos-ir in females. Moreover, when only double-labeled cells are considered (TH-ir and Fos-ir), an increased expression of Fos was only observed in the female consummatory behavior group in anterior PAG. A previous study in males found no effect of copulatory behavior on the number of TH+Fos-ir cells in this structure (Charlier, Ball & Balthazart, 2005). Studies in ferrets also indicated a similar sex difference in Fos immunoreactivity in PAG TH-ir neurons (Wersinger & Baum, 1997). In mammals, a columnar organization of PAG has been proposed based both on morphological and functional criteria (Bandler & Shipley, 1994) and there is evidence for a similar organizational pattern in avian species (Kingsbury, Kelly, Schrock, & Goodson, 2011). In quail, anterior and posterior parts of the PAG correspond to different functional zones proposed by Kingsbury et al., 2011, thus the anatomically discrete patterns of IEG expression in PAG of female quail identified in this study may well be a reflection of this topographical organization.

IEG Induction in Noradrenergic Cell Groups

In the noradrenergic cell groups, LC and SCv, we found no significant difference in Fos induction after expression of both components of sexual behavior. A number of studies have indicated that the noradrenergic system plays an inhibitory role in male sexual behavior (Balthazart, Libioulle, & Sante, 1988; Rodríguez-Manzo & Fernández-Guasti, 1994). However, the present study did not demonstrate a significant increase in Fos-ir in either LoC or SCv suggesting that either these structures were not activated during the performance of the behavior in our testing conditions or more probably that this activation does not result in an induction of the IEG under study.

Serotonergic Nucleus of Raphe Pallidus

The inhibitory role of serotonin in sexual behavior is well established in rodents (Fernandez-Guasti et al., 1992; Lorrain et al., 1997). For instance, an increase in 5-HT concentrations has been documented in the lateral hypothalamic area after ejaculation (Lorrain et al., 1997). The present study found elevated Fos-ir expression in the raphe pallidus only following consummatory sexual behavior in male quail. In agreement with previous findings, the present results are thus consistent with the notion that serotonergic inputs rooted in the raphe pallidus may be responsible for the modulation of the satiation of male sexual behavior.

Implications for Our Understanding of the Functional Organization of the Sexual Behavior Circuit

Studies reported in this chapter supported previous evidence on POM's role as a key integrative site in the male sexual behavior circuit in quail (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013) as is the case in other vertebrate species (Hull, 2011). This brain area seems

to receive the relevant sensory inputs and then projects via the PAG to the brain areas needed to implement the motor output required to engage in both appetitive and consummatory components of male-typical sexual behaviors (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013). Another key component of the regulation of male sexual behavior includes the many projections to the basic sexual behavior circuit such as those by the monoamines and by various neuropeptide systems. Behavioral studies by Beach (1956), Everitt (1990) suggested that appetitive and consummatory aspects of male-typical sexual behavior could be differentially controlled by various aspects of the integrated hypothalamic-limbic circuit that controls sexual performance and motivation. Work completed in quail suggested that there were differences in the control of these different aspects of male sexual behavior within the POM itself (Balthazart et al., 1998) with the more rostral POM being important in the regulation of appetitive behaviors while the more caudal part was important in consummatory aspects of the behavior. In this study we have confirmed the significance of the POM and the closely connected BNST in mediating both aspects of male-typical sexual behavior and we have identified new insights into the monoaminergic modulation of the circuit.

In the current study we have observed that cell groups previously implicated in the control of consummatory aspects of male sexual behavior (Charlier, Ball & Balthazart, 2005) are involved in appetitive sexual behavior as well. So for example, the VTA exhibits gene expression when males are engaged in appetitive as well as consummatory aspects of male-typical sexual behavior as was shown previously (Charlier, Ball & Balthazart, 2005). Similar observations have been made concerning the rostral and caudal PAG. Interestingly, cell groups such as the SN and the noradrenergic cells groups such as the LoC and the SCV that did not exhibit significant IEG expression in association with consummatory behaviors in the previous

study (Charlier, Ball & Balthazart, 2005) also did not for male-typical appetitive sexual behaviors in the current study. These findings suggest that modulatory catecholamine systems seem to be generally involved in the regulation of male-typical sexual behavior, in other words if a particular cell group expresses Fos in association with engaging in one component of male-typical sexual behavior it will also express IEGs in association with the other.

Cells in the Raphe pallidus did not exhibit Fos expression in association with appetitive behaviors but did in association with consummatory behaviors. Thus the indoleamine serotonin unlike the catecholamine systems exhibits a more selective pattern of IEG expression. The number of TPH cells expressing Fos was only elevated when males engaged in copulatory behavior per se. Interestingly, in all our studies there did not tend to be a significant effect if one focused on double labeled cells, i.e. cells labeled with TH that also expressed Fos. The only exception was in females engaging in consummatory sexual behavior where Fos expression increased in TH cells in the posterior PAG. In the Raphe pallidus, the Fos expressing cells correlated with engaging in consummatory but not appetitive behaviors in males also tended to be double-labeled with TPH.

Our studies of gene expression and female sexual behaviors are harder to put in a broader context. One previous study did investigate Fos expression in female quail (Meddle et al. 1999), but much less is known about the circuit mediating the control of the female genitals in mammals or birds (Marson & Murphy, 2006; Ball & Balthazart, 2009). The somewhat preliminary evidence that is available suggests that there is much in common between male and female quail in the circuit that controls genital responses associated with sexual behavior (Wild & Balthazart, 2013). In our study we collected female quail that were each interacting with a male just prior to copulation and in females after they had engaged in sex-typical sexual behavior. Under the

standard laboratory testing conditions female quail often do not exhibit clear indications of appetitive sexual behaviors so in this testing situation we describe them as having visual access to males but do not claim that are actively engaging in appetitive sexual behavior. We found no increase in Fos expression in the visual contact group for female quail; the possible variation in the appetitive state of each female may have contributed to this outcome. For females in the physical contact group an increase in Fos-ir was present in VTA and Anterior PAG but not in POM or BNST after interacting with the male and engaging in copulatory behavior. Thus VTA and PAG seem to exhibit signs of cellular activation in a sexual context in both males and females. VTA is one of the regions involved in the mesolimbic reward circuitry, and had been associated with sexual behavior in mammals. The lack of effects in other nuclei in females requires further studies of the female sexual behavior circuit using different testing conditions for females to interpret them properly.

Even though, findings from this chapter provided valuable evidence concerning the roles of different monoaminergic neurotransmitters in modulation of sexual behavior, it also revealed two serious limitations in relation to the study of sexual behavior in avian species: 1) there is a paucity of behavioral tests to address female appetitive behaviors, and also 2) The exact anatomical location of the nucleus accumbens (Ac), its subdivisions and its function in avian species has been subject to some controversy. Therefore previous studies (Bharati & Goodson, 2006), including this one failed to report findings from this nuclei. The following chapter will address these issues.

Chapter 3 - Localization of the Nucleus Accumbens in Japanese Quail Based on Hodological, Immunohistochemical and Functional Criteria

Rationale

The nucleus accumbens (Ac) is a well-defined ventromedial structure in mammals and is a key part of the mesolimbic circuitry that plays a critical role in regulating motivation and reward. Ac has been credited for evaluating incentive salience and hedonic properties of adaptively significant stimuli, thus there are numerous studies in the mammalian literature addressing the role of this particular nucleus in association with reward mechanisms (Berridge & Kringelbach, 2013). Its structure and function has been best studied in a wide variety of mammalian species (Berridge, 2004), correspondingly there are some studies indicating that the circuits controlling reward mechanisms are evolutionarily conserved among vertebrates (O'Connell & Hofmann, 2011; Weitekamp & Hofmann, 2014). However, there is a paucity of complementary studies in avian species since the exact anatomical location of the Ac, its subdivisions and their functions has not been as well-characterized and remains a somewhat controversial subject (e.g., Bálint & Csillag, 2007; Bálint, Mezey, & Csillag, 2011; Husband & Shimizu, 2011; Reiner et al., 2004).

Avian brain atlases if one focuses on the past 50 years have proposed different anatomical locations for the nucleus: The first avian atlas of the modern era of neuroscience, the pioneering pigeon atlas prepared by Karten & Hodos (1967) localized the Ac at the ventral end of the lateral ventricle. Posterior parts of this putative Ac are now considered to be a component of the lateral part of the bed nucleus of stria terminalis (BNSl) (Aste et al., 1998; Reiner et al., 2004) and also contribute to the ventral parts of the shell region of the Ac as argued by Bálint et al. (2007). The Karten and Hodos pigeon atlas was very influential and their proposed

localization of Ac and the associated nomenclature was adopted by several subsequent avian atlases with some modifications, such as the chicken (*Gallus gallus domesticus*) (Kuenzel & Mason 1988; Puelles et al., 2004; Youngren & Phillies, 1978); the canary (*Serinus canaria*) (Stokes et al., 1974); goose (*Anser anser*) (Felix et al., 1982); quail (Bayle, Ramade & Oliver, 1974); and the zebra finch (*Taeniopygia guttata):* (Nixdorf-Bergweiler & Bischof, 1979). More recently, Boer-Visser, Brittijn & Dubbeldam's (2004) collared dove atlas located the Ac at the most anterior tip of the lateral ventricle, lateral to BNSl. Overall, the Ac depicted in these atlases, corresponds mostly to anterior parts of the BSTl and/or has a smaller volume as compared to recent proposals for its anatomical localization in different avian species (Abellán & Medina, 2009; Bálint & Csillag, 2007; Garcia-Calero, Bahamonde, & Martinez, 2013; Husband & Shimizu, 2011; Roberts, Hall, & Brauth, 2002).

Recent studies provide convergent lines of evidence for a new view of the possible anatomical location of avian Ac: it is adjacent to BSTl and medial striatum (Mst) and dorsal to ventral pallidum (VP) but extends further in a posterior direction as compared to previous proposals. One type of criteria for nuclear definition utilized in these studies is based on the localization of different neurochemical markers localizaed via immunohistochemistry to delineate the borders between Ac and surrounding brain structures, namely the lateral bed nucleus of stria terminalis (BNSl) and medial striatum (Mst). The dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) is abundant in the rat (Schalling et al., 1990) pigeon (Durstewitz, Kröner, Hemmings, & Güntürkün, 1998; Reiner et al., 2004) and chicken (Bálint & Csillag, 2007; Bálint et al., 2011; Csillag, Bálint, Adám, & Zachar, 2008) striatum and putative Ac. Bálint and Csillag (2007) showed in the chicken relatively dense DARPP-32 immunoreactivity in the putative Ac and the surrounding Mst in comparison to BSTl (Bálint &

Csillag, 2007). Additional studies have used markers of calcium binding proteins (i.e. Calbindin 28k, Calretinin) to delineate the borders of Ac and the surrounding regions (Bálint & Csillag, 2007; Husband & Shimizu, 2011; Roberts et al., 2002).

Another line of studies explored the hodology of the putative Ac via tract-tracing methods. These studies demonstrated that the pattern of connectivity of the avian Ac is similar to its mammalian homolog, and includes dense connections to BSTl, hippocampus (Hp), ventral pallidum (Vp), hypothalamus and ventral tegmental area (VTA) (Bálint & Csillag, 2007; Hanics et al., 2012). These limited numbers of studies provide accumulating evidence suggesting an anatomical localization for the avian Ac somewhat distinct from initial proposals (Abellán & Medina, 2009; Bálint & Csillag, 2007; Bálint et al., 2011; Husband & Shimizu, 2011). Even though these studies provide valuable information on the anatomical localization of Ac there are still a number of aspects to these claims that need to be further investigated: (1) a limited number of avian species have been subject to investigation, (2) discrepancies remain on the precise borders of the avian Ac and its sub-regions, (3) and most importantly there is a paucity of studies that investigate the functional similarities between suggested avian Ac and its mammalian homologue. The present study, therefore, aimed to expand the results of the previous studies using Japanese quail (Coturnix japonica), which has been frequently used in research on reward related behaviors, using converging evidence from hodological, immunohistochemical and functional criteria.

To this end, we examined the distribution of the following proteins to histochemically define the Ac and its subregions: the serotonin transporter (SERT), Dopamine- and cAMP-regulated phosphoprotein (DARPP-32), and Calretinin. Subsequently, we examined the hodology of the Ac by stereotaxically injecting a biotinylated dextran amine (BDA) as a

neuroanatomical tracer into the Ac and its putative subregions as delineated per the histochemical results. Drawing on these anatomical findings, we examined the functional role of the avian Ac in response to the performance of rewarding behaviors. For male quail we measured Egr-1-immunoreactivity (ir) in response to socio-sexual interactions. For female quail, we quantified Egr-1-ir in Ac along with the Phonotaxis behavior in response to male conspecific vocalizations, which is hypothesized to be rewarding for the animal.

Materials and Methods

Subjects

A total of 35 male and 10 female Japanese quail (*Coturnix japonica*) were used in the present studies. Japanese quail were obtained from a local breeder (Maryland Exotic Birds). All subjects were housed individually and maintained on a standard 16L:8D cycle to insure that they are reproductively active (Ball & Balthazart, 2010). Animals had food and water available *ad libitum*. All of the experimental procedures were in accordance with Johns Hopkins University Animal Care and Use Committee guidelines.

Brain Extraction and Fixation

Following different experimental conditions listed below, subjects were decapitated and their brains were extracted and immersed in acrolein (Polysciences Inc., CAS 107-20-08). Brains were mildly agitated in acrolein for 3 hours and then ran through 4 successive 15-minute washes of PBS. After the last PBS wash, brains were placed in a 30% sucrose solution for 24 hours or until the brain sank to the bottom of the vial. Brains were then frozen on dry ice and placed into a freezer at -70 degrees C until sectioning. Brains were removed from the -70, sectioned at 40

microns using a cryostat into four series and stored into cryoprotectant. These series were placed at -20 degrees C.

Immunohistochemistry and Antibody Characterization

For each antibody, one series of brain tissue were used from three different male Japanese quail. A standard avidin-biotin horseradish peroxidase (ABC) staining procedure on free-floating, acrolein-fixed tissue was employed, the details of the procedure had been explained in the previous chapter. Table 1 demonstrates the information related to the primary antibodies used in the present study, including the immunogens, host species, catalog numbers and dilutions used.

TABLE 1.
Details of Primary Antibodies Used

Antigen	Immunogen	Host Species, Source & Cat#,	Concentration
SERT	Amino acids 579-599 of rat	Rabbit Polyclonal, Immunostar 24330	1:10.000
Calretinin	Full-length human protein	Rabbit Polyclonal, Abcam ab702	1:5.000
DARPP-32	Bovine DARPP-32	Mouse Monoclonal, custom	1:10.000

DARPP-32 antibody is mouse monoclonal antibody is directed against bovine DARPP-32. This antibody had been developed and tested for its specificity by Hemmings & Greengard, (1986). The distribution of the DARPP-32 immunolabeling was identical to previous reports in Japanese quail (Absil et al., 2001).

Anti- Serotonin transporter (SERT) is a rabbit polyclonal antibody directed against amino acids 579-599 of rats. According to manufacturer's report this antibody recognizes a single band in Western blot and preadsorption controls eliminates immunohistochemical detection in rats.

The Preadsorption controls of this antibody in the present study eliminated immunostaning in both Japanese quail (via 50 μg/ml SERT peptide, Immunostar Cat# 24332). In addition, the observed immunostaining pattern of SERT-ir in auditory forebrain of the Japanese quail in the present study is consistent to the distribution of the same antibody in white-throated sparrows (Matragrano et al., 2012). Similar to previous reports, we also documented the presence of a dense SERT-ir in the pretectal nucleus, the lateral geniculate nucleus and the nucleus taeniae of the amygdala (Matragrano et al., 2012). The calretinin antibody raised against full-length human calretinin protein. This antibody recognizes a single band in Western blots of zebra finches (Pinaud et al., 2008). The distribution of calretinin of Japanese quail striatum is similar to reports, in chicken (Bálint & Csillag, 2007) and pigeon (Husband & Shimizu, 2011).

Retrograde Pathway Tracing and Tracer Visualization

For neuronal tracing adult male Japanese quail were anesthetized using isoflurane and placed in a stereotaxic apparatus. Biotinylated dextran amine (BDA, 3kDa) was stereotaxically injected into the putative Ac as a retrograde neuroanatomical tracer of 6 male Japanese quail, at this molecular weight (3kDa), BDA retrogradely labels cell bodies that send projections to the injection site (Reiner et al., 2004). The skull was open above the target brain region and a Hamilton Neurosyringe was lowered to the desired coordinates, AP:+4, ML±.7, DV:-4.5 . The zero coordinate was taken from the rostral tip of the cerebellum. Upon reaching the desired dorsal-ventral coordinate, 100 nanoliters of BDA was injected using pressure injection. The syringe remained in the brain for 10 minutes and was removed slowly. The skin was sutured,

birds recovered under a heat lamp, and were then returned to their home cage for 14 days; and then their brains were extracted as described above.

BDA was visualized using avidin-biotin horseradish peroxidase (ABC) staining procedure on free-floating, acrolein-fixed quail tissue. Tissue was transferred to PBS and washed for 5 minutes three separate times and then washed in 0.1% sodium borohydride for 30 minutes. After three more 5-minute PBS washes, tissue was incubated for 30 minutes in 3% hydrogen peroxide to block any endogenous peroxidase activity. After three PBS washes, tissue was incubated for 4 hours in ABC. Tissue was washed in PBS and then immersed in sodium acetate for 5 minutes. Tissue was then exposed to diaminobenzidine mixed with Nickel for chromophoric visualization for 4-7 minutes. The reaction was stopped with sodium acetate, tissue was then washed in PBS and remained in PBS until tissue was mounted on gelatin-coated slides. Slides were coverslipped using exposure to successively higher concentrations of ethanol and then exposure to xylene and coverslipped using permount.

Cells immunoreactive for Egr-1 were visualized using the same basic procedure as above. After the sodium borohydride step, tissue was incubated in H_20_2 as above, but mixed with normal-goat serum to prevent non-specific binding of the antibodies, for 1 hour. Tissue was incubated for 48 hours in egr-1 antibody (1:5,000) in PBS-Triton mixed with NGS (2%) at +4° C. Tissue was then immersed in goat anti-rabbit antibody (1:250) for 1 hour, immersed in ABC and then Ni-DAB as above, but for 3 minutes. Tissue was mounted and coverslipped identically as above.

Functional Experiments

Experiment 1: The Effects of Sociosexual Interactions on Egr1-ir in Ac of Male Quail

At the start, all male quail experienced five consecutive days of a five-minute pretest when males and females could interact freely to ensure all of the male quail were sexually active. Five days after the last pretest one male and one female quail were placed at different sides of the experimental chamber (0.60m x 0.20m x 0.20m) (See Figure 3 in chapter 2 for details). Male and stimulus female quail freely interacted for fifteen minutes during which all of the males showed the typical consummatory sexual behaviors. Animals in the control group were placed to the same experimental chamber that did not contain a congener of the opposite sex for the same amount of time.

Experiment 2: The Effects of Conspecific Male Vocalizations on Egr1-ir in Ac of Female Quail

All experimental female quail, were placed to a sound attenuated experimental chamber (1m x 0.90m x 0.90m) (See figure 12) for five consecutive days for 8hrs a day (approximately from 9:00 am to 6:00 pm), with water and food available *ad libitum*. Prior to experimental manipulations we documented that all female quail laid eggs which has been considered as an indicator that they are reproductively active. On the fifth day of testing female quail were observed via a camcorder system. When they were in a specifically defined zone, previously recorded male conspecific vocalizations, crows, were played via speaker for 15 minutes (N=7). For the control condition reverse crows were played (N=6). The phonotaxis behaviors of the subjects were observed during the 15 minute period, 2 of the female quail did not showed any approach behavior they were excluded from immediate early gene analysis. For both male quail

in experiment 1 and female quail in experiment 2 the brains were collected 90 minutes after the onset of the experimental manipulations.

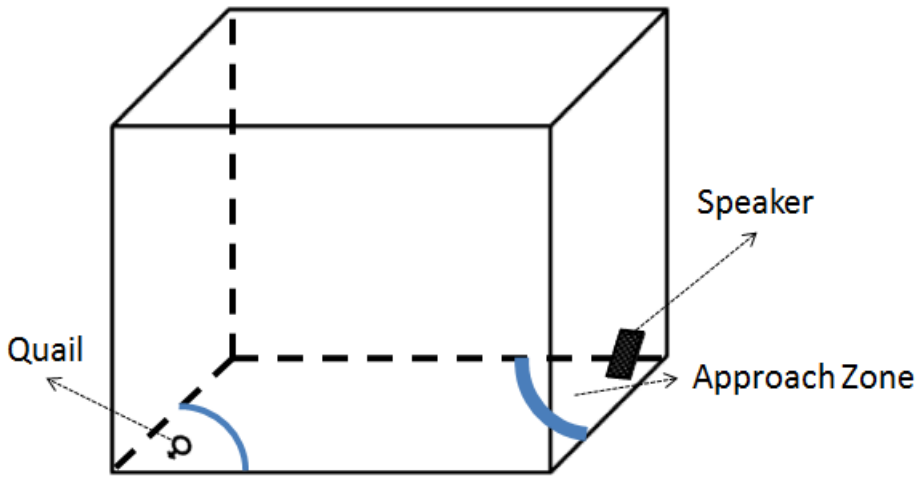

Figure 12. A schematic representation of testing apparatus.

Results

Immunohistochemical Findings:

Figure 13 demonstrates the distribution of the four histochemical markers that helped to elucidate the different subdivisions of the Ac and demarcate Ac from adjacent regions. For instance, the present study documented visibly denser DARPP-32 immunoreactivity in Ac and surrounding Mst in comparison to BSTl, which allowed us to demarcate the border between Ac and BSTl. Similar to DARPP-32, SERT-ir was denser in the Ac compared to BSTl. In addition for quail, SERT-ir was more pronounced in Ac than Mst. Calretinin were distributed heterogeneously within the sub-regions of Ac, higher expression in the shell region than in the core region was documented but no clear cut delineation was present for the putative

subdivisions of this nuclei.

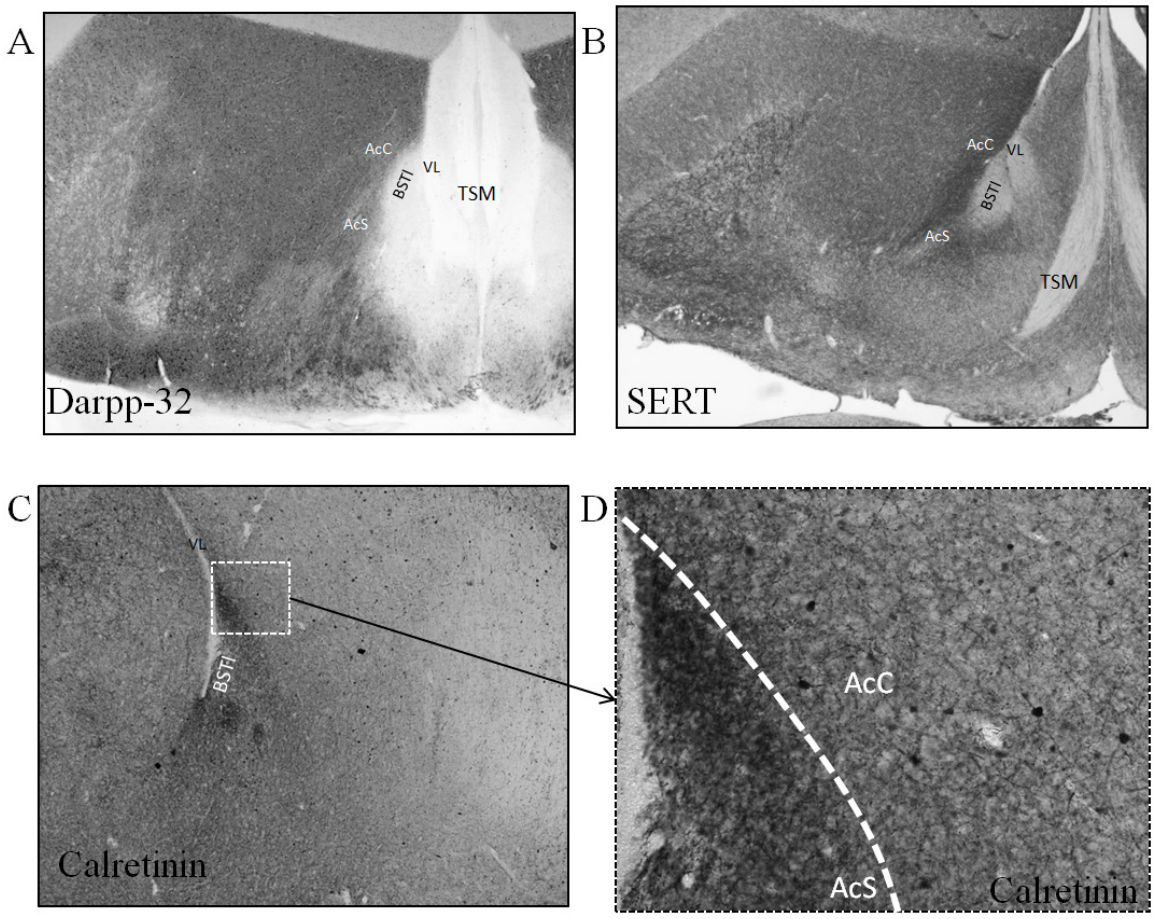

Figure 13. Photomicrographs illustrating the brain regions surrounding nucleus accumbens (Ac) with different immunohistochemical markers. Panel A illustrates the distributions of Darpp-32 and B illustrates the SERT immunoreactivity. Panel C illustrates the distribution of calretinin in core and shell region of the Ac, panel D is a close-up (20X) photomicrograph of Ac, white dotted lines demarcate the borders of the nucleus.

Retrograde Pathway Tracing:

In all 3 animals that have correct BDA injection, BDA positive perikarya demonstrated afferent inputs to Ac from hippocampus (Hp), ventral tegmental area (VTA), BSTl, raphe pallidus (Rp), as well as ventral part of the mesopallium (MVS) and ventral corticoid plate region of mesopallium (MVcp). On the other hand, miss-lesions (n=3) resulted with BDA injection in the surrounding medial striatum (Mst) instead of Ac had a different pattern of BDA immunoreactivity throughout the brain.

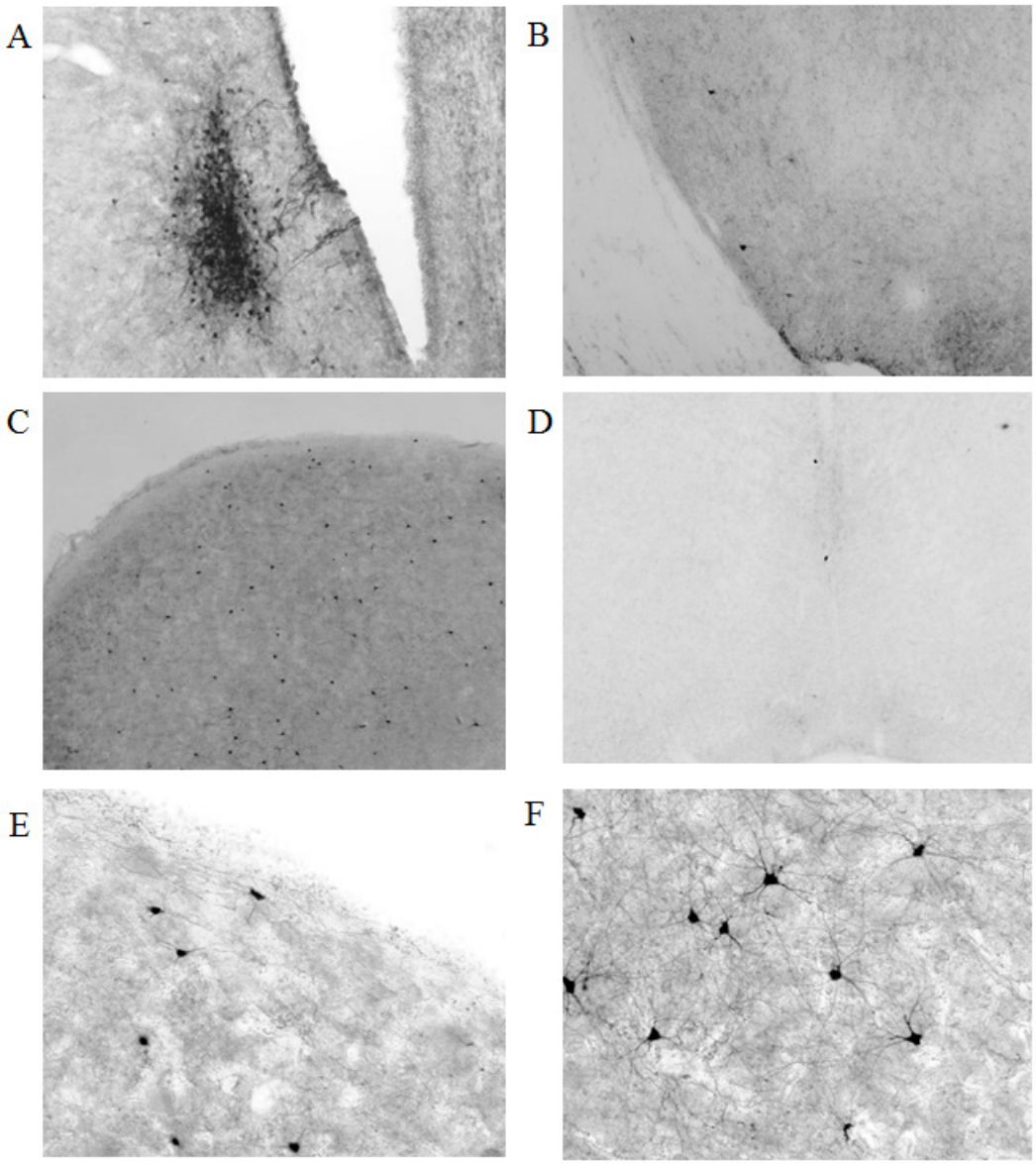

Figure 14. BDA positive perikarya (A) in the injection site of nucleus accumbens (Ac) and retrograde labeled cells in (B) ventral tegmental area VTA, (C) hippocampus (Hp), (D) raphe pallidus (Rp), (E) ventral corticoid plate region of mesopallium (MVcp) and ventral part of the mesopallium (MVS).

Functional Experiments

1. The effects of Sociosexual Behavior on Egr-1-ir in Ac of Male Quail

Figure 15 represent the mean number of Egr-1-ir nuclei in the Ac including its core and shell subdivisions. Independent samples t-test demonstrated that sociosexual behaviors induced a

significant Egr-1-ir in Ac ($t(9) = 3.45$, $p < .05$.). For the subdivisions of the Ac, the significant difference between animals that showed sociosexual behaviors and controls condition was present for in the shell region of Ac ($t(9) = 3.65$, $p < .05$), but not in the core of male Japanese quail ($t(9) = 1.73$, $p > .05$).

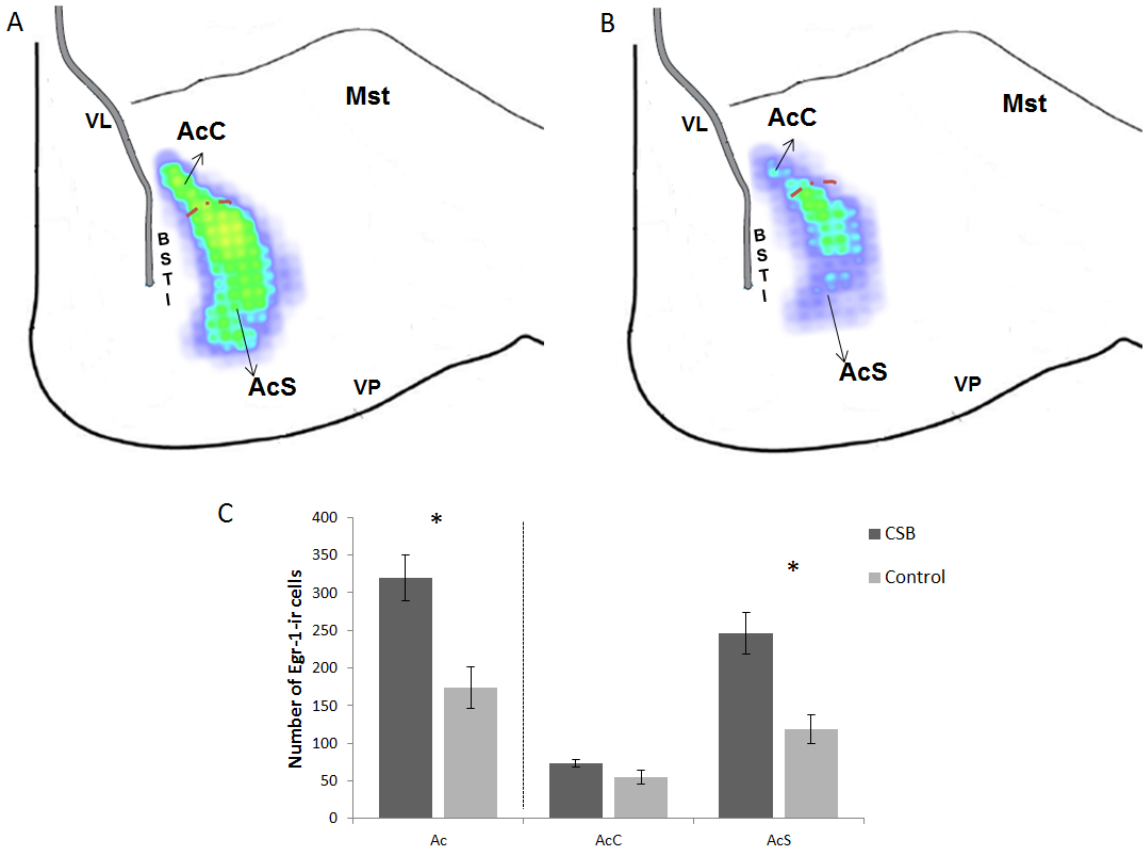

Figure 15. Heat map of the mean Egr-1 immunoreactivity (ir) for male quail either (A) tested in control condition or (B) engaged in socio-sexual interactions. Mean number of Egr-1-ir cells in Ac along with the core and shell regions of the nuclei of the male quail (C). Error bars represents the standard error of the mean.

Experiment 2: The Effects of Conspecific Male Vocalizations on Egr1-ir in Ac of Female Quail

Figure 16 represents the mean number of Egr-1-ir nuclei in the Ac including the core and shell subdivisions. Independed samples t-tests yielded a significant Egr-1-ir in Ac for females that exposed to conspecific male vocalizations and showed the approach behavior when compared to control condition ($t(9) = 2.27$, $p < .05$). For the subdivisions of the Ac, the significant difference between animals crow and reverse crow condition was present for in the shell region of Ac ($t(9) = 2.28$, $p < .05$), but not in the core of female Japanese quail ($t(9) = 1.15$, $p > .05$). The histogram on Panel D of figure 16 demonstrates that female quail exposed to male conspecific vocalization, spent more time in close proximity to speaker when compared to the females exposed the same stimulus in reverse ($t(9) = 2.09$, $p < .05$)

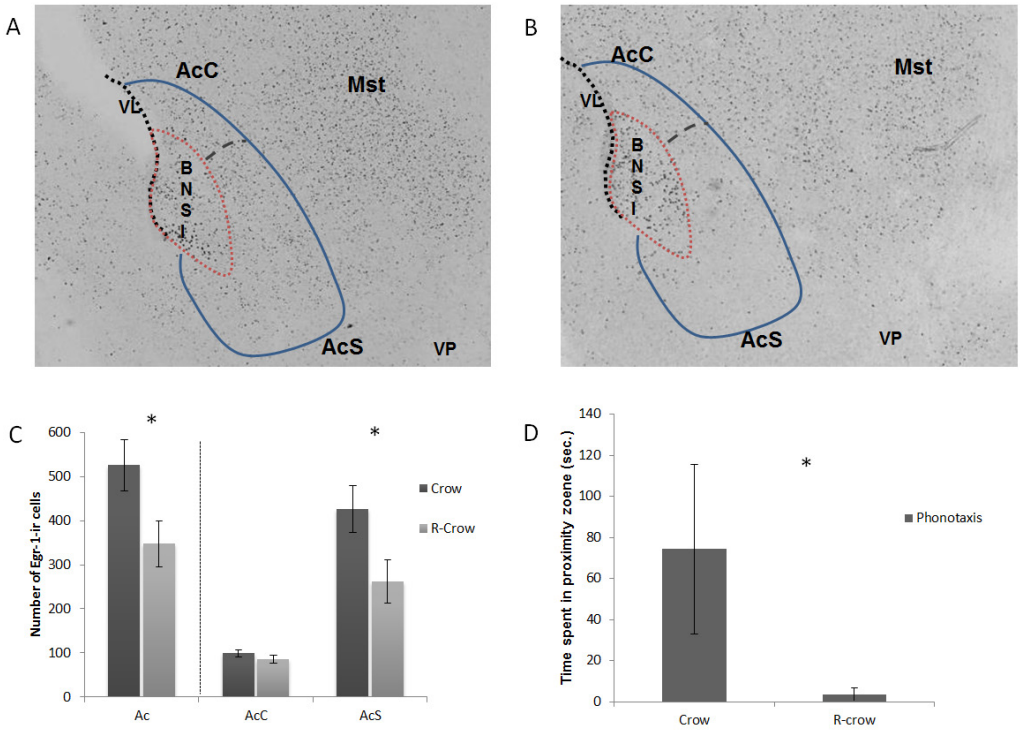

Figure 16. Photomicrographs of the Egr-1 immunoreactivity (ir) for female quail either (A) engaged in socio-sexual interactions or (B) tested in control condition. Mean number of Egr-1-ir cells in core and shell regions of Ac of the male quail (C). (D) Mean number of seconds female quail spent in close proximity to speaker.

Discussion

This study provides significant new information to increase the precision of our understanding of the localization of the nucleus accumbens in avian species, especially for Japanese quail. Overall, our localization of Ac via immunohistochemical criteria is consistent with and extends some recent avian studies that have proposed homologies between structure in the avian brain and the mammalian nucleus accumbens (Bálint & Csillag, 2007; Bálint et al., 2011; Husband & Shimizu, 2011). The afferent hodology of Ac described in the present study, based on the retrograde BDA tracer in Japanese quail is congruent to its mammalian homologue (Heimer, 2003) . In addition, the current study aimed to investigate if avian Ac has functionally distinct subdivision, core and shell, as in concordance with the mammalian species. A functional difference based on variation in the expression of immediate early genes suggests that differentiation of the subdivision may also be critical for avian species. These results collectively contribute to a growing body of work suggesting that the initial proposals for the location of Ac need to be refined and updated (Karten and Hodos 1967 and others).

Immunohistochemical Findings

Here we characterized the distribution patterns of three markers that we hypothesized would provide insight into the organization of the avian accumbens based on their distribution in mammals: DARPP-32, SERT, and Calretinin. These markers did indeed enable us to delineate the different parts of the borders of Ac as well as the subdivision of the nucleus. DARPP-32 functions as an integrator of neurotransmission in dopaminoceptive neurons, with predominantly dopamine D1 subtype receptor expression (Svenningsson et al., 2004). Consequently, DARPP-32 is ample in the rodent Ac, one of the major targets of dopaminergic transmission (Perez & Lewis, 1992; Schalling et al., 1990). A similar pattern had been documented also in chickens

(Bálint & Csillag, 2007; Csillag et al., 2008) and European starlings (Pawlisch & Riters, 2010). Bálint and Csillag (2007) showed in the chicken dense DARPP-32 immunoreactivity in the putative Ac and the surrounding Mst as compared to the adjacent BSTl thus enabling the researchers to define the border between these two adjacent nuclei (Aste et al., 1998). However, as noted by the authors, DARPP-32 immunohistochemistry did not outline the presumptive borders between Ac and Mst or the subregions of the Ac (Bálint & Csillag, 2007). In the present study, DARPP-32 immunoreactivity also clearly delineate the borders between Ac and BSTl for Japanese quail, however, as shown by Bálint and Csillag (2007), no clear differences in immunoreactivity patterns were documented within the subregions of Ac or the surrounding Mst. This replication was a key observation in our investigation of the avian Ac.

Calcium-binding proteins known to be expressed differently in the core and shell region of the Ac in mammals (Brauer et al., 2000; Bubser, Scruggs, & Young, 2000; Härtig et al., 2003; Tan, Williams, & Zahm, 1999). Also a core-shell difference has been documented for Calbindin (Bálint & Csillag, 2007; Garcia-Calero et al., 2013), parvalbumin (Husband & Shimizu, 2011; Roberts et al., 2002) and Calretinin (Husband & Shimizu, 2011) in different avian species. Interestingly, in mammalian studies these different calcium binding proteins expressed more in core region of the Ac (Brauer et al., 2000; Bubser et al., 2000; Hartig et al., 2003; Tan et al., 1999), however in all of the avian studies mentioned above, including the present study markers of the calcium-binding proteins expressed more densely in the shell region (Garcia-Calero et al., 2013). Similialy we documented relatively more expression in the putative shell region of the Ac for calretinin in this chapter. In addition, our ongoing studies in European starlings (*Sturnus vulgaris*) also had a similar core-shell dichotomy in distribution of other calcium binding proteins.

Serotonin modulates a wide range of sensory functions and behaviors. Therefore, in mammals, SERT is widely distributed in the brain (Kretzschmar et al., 2003), and densely present in the Ac (Brown & Molliver, 2000). SERT-ir was broadly expressed throughout the brains of quail, including the hippocampus, hypothalamus, striatum, auditory and visual processing areas, and the periaqueductal gray. The pattern of immunoreactivity was similar as that described in other avian non-songbirds (Challet et al., 1996; Cozzi, Viglietti-Panzica, & Aste, 1991), as well as songbirds (Matragrano et al., 2012, 2013). However, none of these studies investigate the distribution of SERT in the surrounding regions of Ac. In the present study, SERT-ir clearly delineates the border between Ac, BSTl and Mst.

Overall, however, it is our opinion that none of these markers, when used independently are sufficient to establish homologies with a high level of confidence. However, together they are useful tolls to delineate the borders of the Ac.

Tracing Findings

Based on tract tracing studies in mammals, key criteria for determining the location of the Ac subdivisions are based on hodological parameters. Concordant with the mammalian Ac, quail receives projections from the VTA, Hp, BSTl, Rp, MVcp, and MVS (Delfs et al., 1998; French & Totterdell, 2002). Importantly, injections made outside of the Ac in the surrounding Mst demonstrated different retrograde labeling patterns, like having BDA-positive perikarya present in the SN but not in VTA. These hodological results are collectively in line with work in mammals and pigeons (Delfs, et al., 1998; Bálint & Csillag, 2007; French & Totterdell, 2002).

In mammals, the connections between VTA and Ac are studied extensively, and implicated in reward related behaviors (Berridge, 2004). Here we demonstrated same circuitry is

present in the mammalian brain. Subsequent chapters in the present dissertation will assess if the parallels in hodology also corresponds to functional similarities. Likewise, the functional connectivity between Ac and hippocampus has been subject to ample investigation in mammals. For instance, appetitive spatial context condition could be acquired only if these connections are intact (Ito et al., 2008). There are no complimentary functional studies in avian species but the present study demonstrated that there are dense hippocampus-Ac projections similar to reports on mammals.

In addition, we documented BDA immunopositive perikarya in the ventral part of the mesopallium (MVS) and ventral corticoid plate region of mesopallium (MVcp). Previously, based on hodological and histochemical evidence these regions argued to the functional equivalent of the mammalian prefrontal cortex (Merzger, et al., 1998). In addition, in mammals, the functional hodology of prefrontal cortex, Ac, ventral pallidum and mediodorsal thalamus has been documented as the limbic cortico-striato-thalamo-cortical loop which is involved in regulation of affective and motivational responses. Husband and Shimizu (2011) argued that the inputs from the mesopallium to Ac in avian species suggest that a similar circuitry might exist in avian species. However, these proposals are yet to be tested in avian species.

Egr-1 Induction in Ac of Male Quail and Female Quail

Ac is one of the key structures of the mesolimbic reward circuitry, and it has been meticulously studied in mammalian species. For instance, in vivo microdialysis studies found an increase in extracellular levels of dopamine in nucleus accumbens of copulating male rats in comparison to other locomotor activities (Damsma et al., 1992). In addition, both sexual

behavior and sex-related environmental cues enhanced immediate early gene reactivity in dopaminergic VTA neurons and nucleus accumbens neurons (Balfour, Yu, & Coolen, 2004). Moreover, in rats 6-hydroxydopamine (6-OHDA) lesions cause dopamine depletion in the nuclues accumbens which reduced the sexual araousal related to the remote cues from females (Liu, Sachs, & Salamone, 1998). Overall, the role of Ac in mammalian sexual behavior is well documented (Blackburn, Pfaus, & Phillips, 1992) however, there has been a paucity of studies for avian species. The inconsistent identifications and controversies about the anatomical localization of the Ac have been a limiting factor for studies aiming to investigate the functional role of Ac in motivated behaviors in avian species. For example, in our previous attempts, which relied on the localization of Ac provided by the available avian brain atlases, we failed to detect a significant increase in immediate early gene-ir in association consummatory and appetitive sexual behaviors in quail (Iyilikci et al., 2014). After identifying the nuclei with histochemical and hodological markers, we documented a significant increase in Egr-1 immunoreactivity in Ac in response to sociosexual interactions.

We also expand our findings to female quail. Females in many species invest more care and resources in their offspring than males do and therefore tend to be much more selective in terms of which individuals they mate with. However, there is to this day a relative scarcity of studies investigating the behavioral, neural and hormonal correlates of such behaviors in females of Japanese quail. There are a limited number studies that indicate female quail has also show mate choice behaviors. For instance, female quail prefer males that they've seen copulating with other females and avoid males with aggressive tendencies (Persaud & Galef, 2003; White & Galef, 1999) . In addition, Goodson and Adkins-Regan (1997) showed greater female approach, crouching beside, and jumping on speakers that played back male separation crows than those

that played the same crows backwards. Males, in contrast, showed no significant difference between the crows played forwards and backwards. Such behaviors have been considered as signs of sexual motivation in female Japanese quail. Here we also demonstrated male crows induce an approach behavior towards the speaker (Phonotaxis), but the same physical stimulus played backwards it had no effect on females' behavior. Among the seven birds that exposed to male conspecific vocalizations, five of them demonstrated the phonotaxis behavior and had an elevated Egr-1 immunoreactivity in the shell region of Ac. This suggests that, this paradigm is an effective measure to quantify female sexual motivation.

Overall, the serious of experiments presented in this chapter contribute to a growing body of work suggesting a new anatomical location for Ac. In addition, this study was first to demonstrate that shell region of Ac is implicated in sexual behaviors of male and female quail.

Chapter 4 - Dopamine Depletion in the Medial Preoptic Nucleus and Nucleus Accumbens Transiently Impairs Sexual Behaviors in Male Japanese Quail

Rationale

There is a large body of the evidence showing that dopamine is implicated in the regulation of sexual behaviors (Will et al., 2014) . In the previous chapters we have reviewed the literature on systemic admirations of dopamine agonists and antagonists and their facilitatory and inhibitory roles respectively, along with the Ac and POM specific dopamine interventions (also reviewed in Dominguez & Hull, 2005).

It has been argued that dopaminergic inputs to Ac function to attribute incentive salience to all rewarding stimuli, e.g. food, water, variety of social stimuli, drugs of abuse (Berridge & Robinson, 1998; Kelley & Berridge, 2002). In mammals, several studies have also investigated the specific involvement of Ac in the regulation of sexual behaviors as an integral part of the study of general reward mechanisms. For example in rats, amphetamine-induced behavioral sensitization increased extracellular dopamine levels in Ac during sexual behaviors along with the augmentation of sexual behaviors (Fiorino & Phillips, 1999). Microdialysis studies demonstrating precopulatory increase in DA levels in Ac (Damsma et al, 1992; Fiorino, Coury, & Phillips, 1997) in particular, suggest that DA in Ac functions to attribute incentive salience to a sexual stimulus. Despite the surging interest on the functions of Ac in rewarding behaviors, including natural rewards such as sexual behavior, there has been very limited number of complementary studies investigating the role Ac in association with sexual behaviors in avian species. To address this, in the previous chapter we have characterized the anatomical location of Ac, and have demonstrated how sexual behavior can induce Fos-ir in the shell region of the nucleus. One of the aims of the present chapter is to further elucidate the role of DA in Ac

specifically on appetitive and consummatory aspects of sexual behaviors by depleting the dopaminergic inputs to Ac. Appetitive and consummatory behaviors have rarely been investigated in tandem in any species and certainly not in an avian species such as quail that exhibit such clear delineations between these two dimensions of the behavior.

We've also noted that the POM has been shown to contribute to appettitive sexual behaviors, along with it's major role in consummatory sexual behaviors. For example, in male Japanese quail an increase in the extracellular dopamine has been documented in males when a conspecific female is presented, more interestingly, this increase was not present in the animals that did not showed subsequent consummatory sexual behaviors (Kleitz-Nelson, Dominguez, & Ball, 2010). This study indicated that DA in POM is critical for initiation of sexual behaviors. In the present chapter, we are aiming to elucidate further the role of dopamine in this nucleus by depleting the dopamine in POM.

In general, this chapter is set to address two primary questions: Are the (1) incertohypothalamic DA inputs to the POM and (2) mesolimbic DA inputs to the Ac necessary for appetitive and consummatory sexual behaviors in male Japanse quail? To this aim, we stereotaxically injected a catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA), into the POM and Ac, and subsequently investigated the different aspects of sexual behaviors.

Materials and Methods

Subjects

A total of 30 experimentally naïve adult (~10 weeks old) male (21) and female (9) Japanese quail (*Coturnix japonica*) were used in the study. Female quail were not subject to any manipulations only used as stimuli for the behavioral tests. All subjects were maintained on a

standard 16L/8D cycle at approximately 22°C and had food and water available *ad libitum*. Male quail were housed in individual cages throughout the experiment, whereas female quail were housed in groups of 5. All of the experimental procedures were in accordance with Johns Hopkins University Animal Care and Use Committee guidelines.

Experimental Procedures

Male Japanese quail were randomly assigned to one of 3 experimental conditions: Bilateral 6-OHDA injections into POM, Ac, or bilateral sham injections (Either in POM or Ac). Before the start of experimental procedures all male quail were tested once, to confirm that they show stereotypical sexual behaviors. Experimental procedures started with a test of appetitive and consummatory sexual behavior 1hr before the 6-OHDA or sham injections to establish base line levels for sexual behaviors. These behaviors were also monitored 5hr, 24hr, 1 week, and 2 weeks after the surgery. Brains were collected two weeks after the surgery on the last testing day (see figure 17).

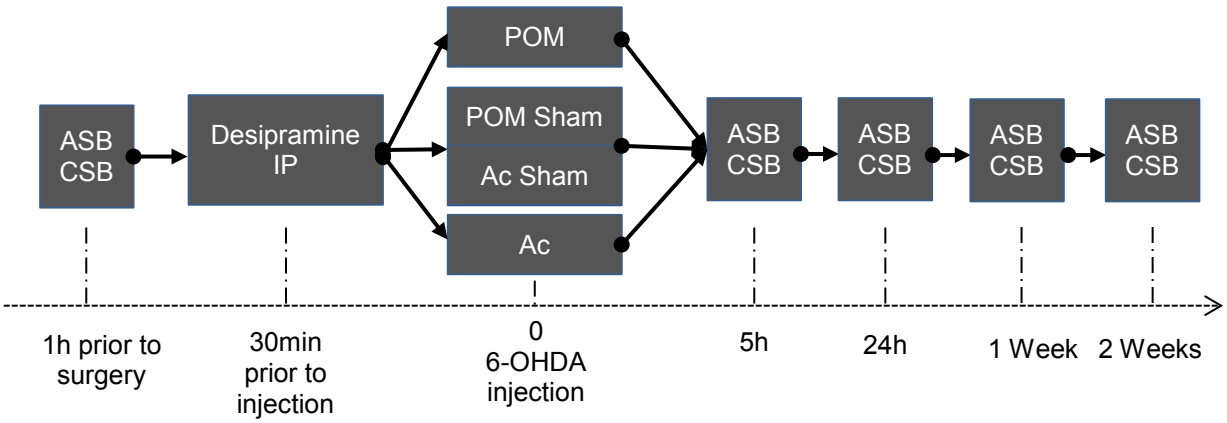

Figure 17. A schematic representation of experimental time line indicating days of appetitive (ASB) and consummatory (CSB) sexual behavior testing, and timeline of surgical procedures.

Apparatus and Behavioral Testing

Behavioral testing took place in an arena that consisted of three compartments separated by two transparent sliding panels. First a female quail is placed in a corner of the arena visible through transparent Plexiglass doors. Subsequently a male quail is placed at the opposite end. Thirty seconds after the placement of the male quail, the first transparent door was removed and the latency of approach to the female quail behind the second Plexiglas glass door is documented as a measure of appetitive sexual behaviors. Two minutes after removal of first door second door is removed and male and female quail were allowed to interact freely to test consummatory sexual behaviors namely the frequency of neck-grabs (NG), mount attempts (MA), mounts (M) and full cloacal contact movements (CCM).

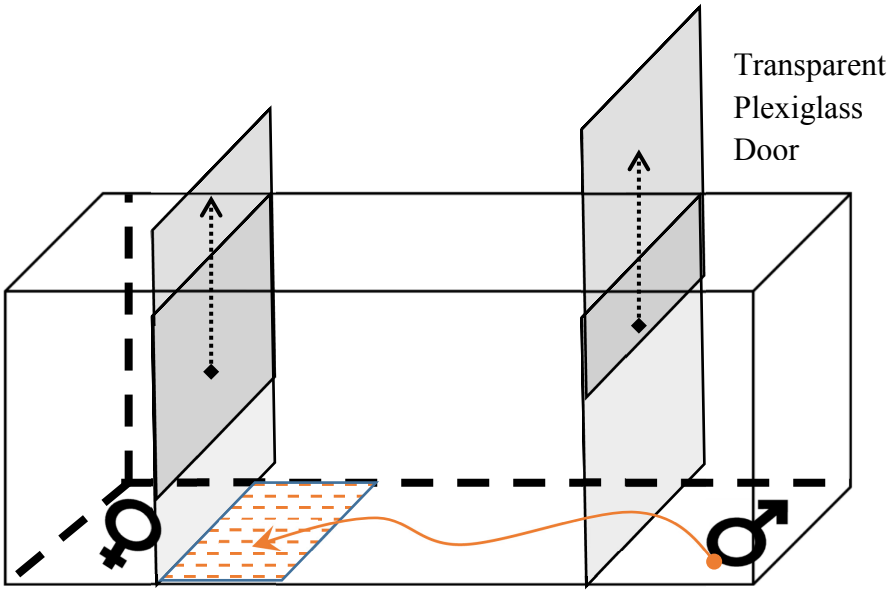

Figure 18. A schematic representation of testing apparatus.

Surgical Procedures

6-OHDA is toxic to both dopaminergic and noradrenergic cells because the transporters these two neurotransmitter have high affinity to 6-OHDA. Once taken into the neuron, 6-OHDA related oxidization causes cytotoxicity and alters the functioning of the effected neurons (Tieu, 2011). In an effort to enhance the dopamine-selectivity of the lesions, an intraperitoneal injection of the noradrenergic reuptake inhibitor, desipramine (25 mg/kg, i.p.), was administered approximately 30 minutes prior to the delivery of 6-OHDA. Prior to surgery quail were first deeply anesthetized with isofluorane gas and then placed in a Kopf stereotaxic apparatus. 6-OHDA was administrated stereotaxically at a concentration of 2µl [10 µg/µl] for POM and 1.5 µl for Ac dissolved in sterile saline (0.9%) solution containing ascorbic acid (0.2%, pH 7.4). A lower dose was assigned to Ac due to high mortality rates associated with this procedure. Sham groups were injected with 2µl of sterile saline (0.9%) solution containing ascorbic acid (0.2%, pH 7.4) into POM and Ac. The coordinates of the POM and Ac were as follows: Ac targeted anteroposteriorly (AP) + 3 mm from ear bars, mediolaterally (ML) ±0.8 from the midline and dorsoventrally (DV) −4 mm from dura. POM targeted anteroposteriorly (AP) + 2 mm from ear bars, mediolaterally (ML) ±0.6 from the midline and dorsoventrally (DV) −4.5 mm from dura.

Fixation and Immunohistochemistry

On the last day of testing subjects were decapitated and their brain dissected out of the skull. The brains were placed into acrolein (5% in phosphate buffer 0.1 M saline) for 3 hours, washed four times in PBS (15 min) and cryoprotected in 30% sucrose for 24 h at 4 °C. The brains were then frozen on dry ice and stored at −70 °C until used. All brains were cut at 35 µm in the coronal plane using a cryostat at –20°C and sections were collected in four series.

TH expression was then visualized by immunohistochemical procedures with the Avidin Biotin Complex (ABC) technique. Three rinses with 0.01 M PBS containing 0.1% Triton X-100

were performed between each step. First, sections were incubated for 60 min in 0.3% hydrogen peroxide and in 20% normal goat serum to remove endogenous peroxidase and decrease non-specific binding. This step was followed by Avidin-biotin blocking (Vector SP-2001) for 15 min, to block possible biotin binding sites in the tissue. Then sections were incubated in the primary monoclonal TH antibody (1:10,000, Immunostar AB22941), for 48 hours. Afterwards, sections were incubated for 60 min in goat anti-mouse serum. The antibody-antigen complex was localized using the avidin-biotin complex method performed with a Vector Elite Kit (ABC Vectastain Elite PK-6100, Vector Laboratories PLC) and finally, the peroxidase enzymatic activity was visualized with DAB (3,3'diaminobenzidine tetrahydrochloride) intensified with Nickel ammonium sulfate and chloride. Reactions were terminated by several rinses in PBS and sections were mounted and coverslipped.

The location of the lesion site was verified via the pattern of TH immunoreactivity documented. Decreases in TH immunoreactivity were observed in the lesioned birds. All the brains showed the correct positioning of the injection within the POM and Ac were included in the study. Nine brains that were miss or largely lesioned excluded from all behavioral and brain analysis. Subsequently, TH immunoreactivity was calculated using NIH-produced image analysis software (ImageJ). Program was calibrated using a step tablet, gray scale values converted to OD units using the Rodbard function. Than images were converted to 8-bit and mean optical density (OD) associated with Ac, POM, and medial striatum (Mst) was calculated. An independent samples t-test was employed to assess OD differences between sham and 6-OHDA conditions.

Results

In figure 19 photomicrographs illustrates the relative reduction in TH immunoreactivity for the 6-OHDA and Sham treated animals. A significant decrease in optical density was documented between 6-OHDA and Sham injected animals in the POM ($t(14)= 3.82$, $p < .05$) and Ac ($t(16)= 5.43$, $p < .05$) where the 6-OHDA was injected, but not in the neighboring medial striatum (Mst) ($t(16)= 0.78$, $p > .05$) (See figure 19).

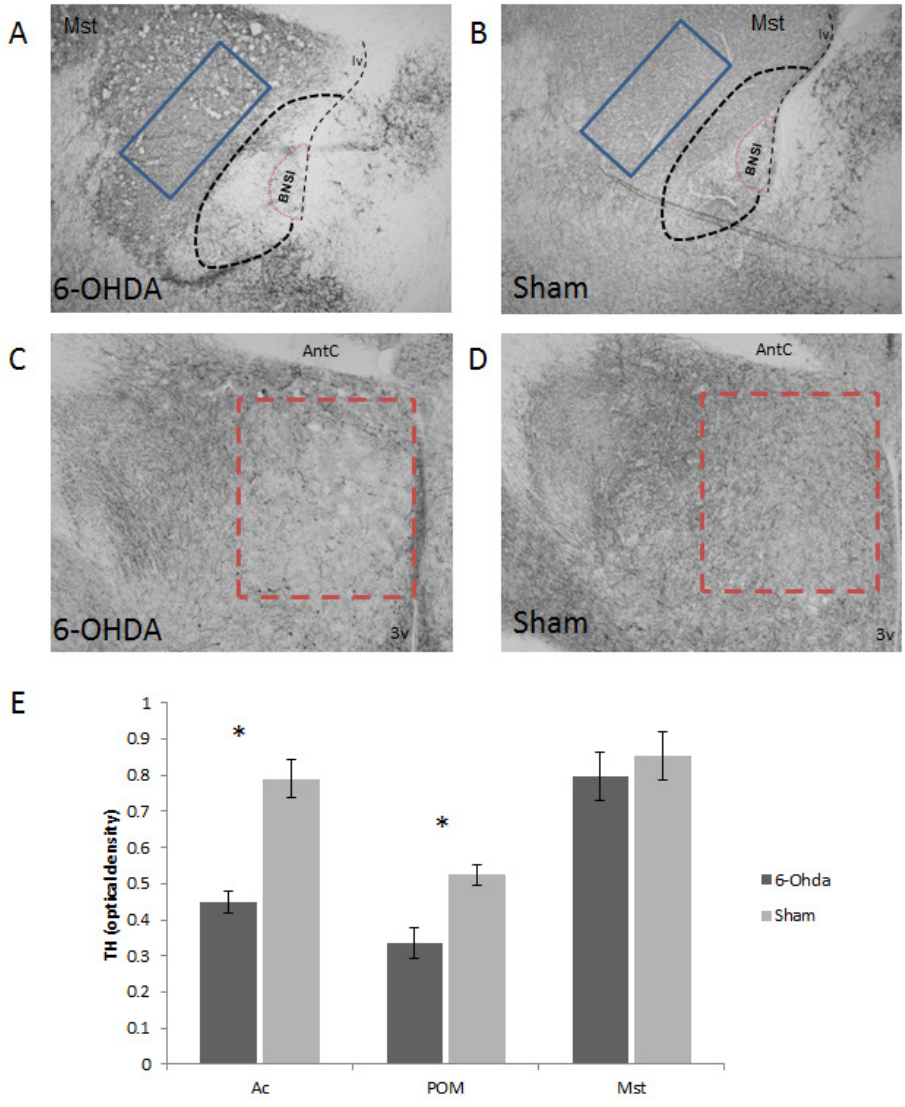

Figure 19. Photomicrographs illustrating the TH immunoreactivity in the: Ac, POM, and part of Mst in 6-OHDA and Sham treated animals. Panel A and B illustrates TH immunoreactivity in the nucleus accumbens (Ac) (doted black lines) and medial striatum (Mst) (blue lines) of 6-OHDA (A) and Sham (B) treated animals. Panel C and D illustrates TH immunoreactivity in the POM (doted orange lines) of 6-OHDA (C) and Sham (E) treated animals. The histogram in panel E represents the optical density of 6-OHDA and Sham lesioned animals.

Behavioral Data

Appetitive behaviors were tested in five sessions: 1hr before and 5hr, 24hr, 1 week, and 2 weeks after the surgery for all three groups. A mixed-design analysis of variance (ANOVA) indicated a significant difference between testing sessions ($F(4, 36) = 20.22$, $p<.05$, corrected for non-sphericity with Greenhouse-Geisser). In addition, there was a significant session and experimental group interaction ($F(8, 36) = 5.88$, $p<.05$, corrected for non-sphericity with Greenhouse-Geisser). Post hoc analysis of LSD yielded a significant difference between sham operated animals and, Ac ($p=.001$) and POM ($p=.005$) lesioned animals, whereas this difference was not significant between Ac and POM ($p=.65$). For further analysis of the data, we also did ANOVAs comparing all the experimental groups for each day. There were no significant differences present for sessions 1hr prior to, 1 week or 2 weeks after the surgery. Conversely, a significant difference present in the session 5hrs ($F(2, 9) = 7.03$, $p<.05$), and 1 day after the surgery ($F(2, 9) = 8.91$, $p<.05$). Post hoc analysis of LSD yielded a significant difference between Sham and 6-OHDA treated conditions for the session that took place 5hrs after the surgery: Ac ($p=.010$) and POM ($p=.014$). No significant difference was documented between Ac and POM ($p=.970$). Similarly, for day 1 post hoc analysis of LSD showed a significant difference between Sham and 6-OHDA treated conditions: Ac ($p=.003$) and POM ($p=.016$). No significant difference was documented between Ac and POM ($p=.519$) (See figure 20 panel A)

Figure 20 panel B presents the mean number consummatory sexual behaviors performed by the male quail in five sessions: 1hr before and 5hr, 24hr, 1 week, and 2 weeks after the surgery for all three groups. A mixed-design analysis of variance (ANOVA) yielded a significant difference between testing sessions ($F(4, 36) = 6.775$, $p<.05$). In addition, there was a significant session and experimental group interaction ($F(8, 36) = 2.645$, $p<.05$,). Post hoc analysis of LSD

showed no significant differences among the different experimental conditions. ANOVAs comparing the three experimental groups for each testing session did not found a significant difference among groups for the sessions 1hr prior to, 1 week or 2 weeks after the surgery. On the other hand, a significant difference was documented for the session 5hrs ($F(2, 9) = 6.866$, $p<.05$), and 1 day after the surgery ($F(2, 9) = 6.444$, $p<.05$). For testing session that took place 5hrs after drug administration post hoc analysis showed a significant difference between control and the two experimental groups: Ac ($p=.019$) and POM ($p=.009$). This difference remained significant between sham and 6-OHDA treated animals, Ac ($p=.010$) and POM ($p=.024$) groups, on the following day.

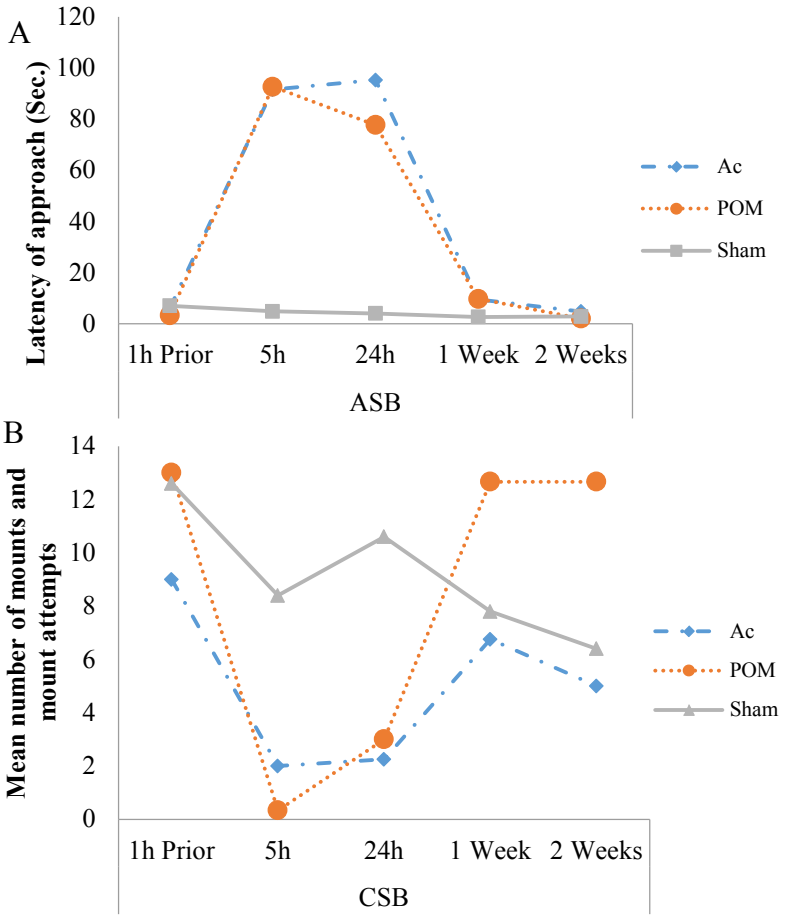

Figure 20. (A) Latency of first sexual approach within 2 min quantification period for 6-OHDA treated Ac and POM and sham treated controls. (B) Frequency of mount attempts and mounts within 2 min quantification period.

Discussion

Animals exposed to 6-OHDA exhibited a rapid impairment in both aspects of sexual behavior and this impairment persisted for 5 hr and 24hr after 6-OHDA injections in both POM and Ac compared to sham injections. However, there was complete recovery of these behaviors 1 week after surgery.

Behavioral Recovery Following the Surgery

One interesting finding of the present chapter is the behavioral recovery of all sexual behaviors one week following 6-OHDA lesions, despite the documented fiber density decreases in both of the areas two weeks after the surgery. Consistently to our results there are several studies, also using 6-OHDA treatment in mPOA, failed to report any behavioral impairments after 3 days (Bitran et al., 1988) or even 24 hours (Bazzett et al., 1992) after surgical manipulations. In other studies, the impairment of sexual behaviors was observed to persist for 12 days and recovered after 16 days, and the impairment was present even in the animals that did not had a significant reduction in TH-ir in the lesion site (Dhawan, Kumar, Govindaraju, & Raghunathan, 1998). In addition high performance liquid chromatography (HPLC) analysis yielded no change in a metabolite of the dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC) levels, and a minor decrease in dopamine levels 3 days after 6-OHDA administration (Bitran et al., 1988). Bitran et al., (1988) argued that one possible way of explaining the discrepancy between decreased fiber density, and steady dopamine levels may be the increased metabolism of the surviving neurons to compensate for the impairments in neighboring DA fibers. In support of this view a time course study on 6-OHDA lesioned documented higher levels of DA efflux in the striatum 3 weeks after surgery when compared to 4 days after surgery (Robinson, Mocsary, &

Camp, 1994). Above mentioned evidence indicates that the extracellular dopamine recovers couple days after the surgery, which is in concordance with the behavioral recovery documented in the present chapter. However it is important to note that the present study did not aim to investigate the recovery in brain or behavior, thus there were no attempts to address this issue.

Dopamine Depletion in Nucleus Accumbens

Results of immunohistochemical analyses of the distribution of TH in brains collected two weeks after injection, demonstrated a 43% decrease in TH-ir fibers density within the Ac (when compared with the mean optical densities of the tissues of control males), indicating an impairment of dopaminergic inputs to this nucleus. This impairment resulted in a sharp disruption of both appetitive and consummatory sexual behavior transiently.

Dopaminergic inputs to Ac are commonly implicated in appetitive behaviors, and yet same inputs suggested having minimal, if any, effects on consummatory aspects of rewarding behaviors (e.g.in feeding behavior Ikemoto & Panksepp, 1996). This dissociation has also been documented for sexual behaviors. For example in rats, 6-OHDA lesions in Ac caused an impairment in sexual araousal related to the remote cues from females, however its effects in consummatory sexual behaviors was negligable (Liu, Sachs, & Salamone, 1998). In addition, DA antagonist (haloperidol) administration into Ac disrupted the appetitive sexual behaviors, but caused minor impairment of copulatory behavior (Pfaus & Phillips, 1991). Pfaus & Phillips (1991) argued that both aspects of the sexual behavior were affected by interruption of DA functions in Ac, but the measures of appetitive behaviors were more sensitive to experimental manipulations.

The marked decrease in the appetitive behaviors in the present chapter is in concordance with the mammalian studies underlying the importance of DA in Ac in relation to motivational aspects of sexual behavior. However, we also documented substantial impairment in consummatory sexual behavior in this study. A possible explanation for this result might reside in differences in testing conditions. Under normal conditions a male Japanese quail would approach to a female within couple seconds and exhibits stereotypical consummatory behaviors, and this behavioral sequence may be as brief as 4 seconds and can be repeated numerous times within in minutes (Hutchison, 1978). Since males are highly motivated to engage in sexual behaviors, usually testing paradigms used in Japanese quail are short. The 270 second testing might not enough time allow males to approach to female in a large arena when their appetitive behaviors are impaired. In addition, it is important to note that animals with 6-OHDA lesions did not have a complete loss of consummatory sexual behaviors. When compared, 6-OHDA lesions in Ac resulted with a 64% decrease in consummatory sexual behaviors whereas animals with POM lesions had 97% impairment consummatory sexual behavior between pretest and first testing after surgery. Also other studies that found a clear dissociation between appetitive consummatory behaviors, for different manipulations, argued that the small testing space used in their study triggered consummatory behaviors reflexively due close proximity of the female (Cornil, Ball, & Balthazart, 2015). In our study since males have to approach to the female quail the impairment in the consummatory sexual behaviors might be secondary to loss of appetitive sexual behaviors.

Dopamine Depletion in Medial Preotic Nucleus

In the first chapter we've reviewed the evidence related to involvement of DA in consummatory sexual behaviors. (e.g. Hull et al., 1986; 1995; Pfaus & Phillips, 1991). In

addition, rodent studies have documented an increase in the dopamine concentrations in mPOA of males when presented with a receptive female (Will et al., 2014). For instance, in vivo microdialysis studies demonstrate an enhancement in extracellular DA activity during precopulatory exposure to female conspecifics in Preoptic area of the male quail (Kleitz-Nelson, Dominguez, & Ball, 2010), suggesting that DA is also involved in the motivational aspects sexual behavior.

The robust impairment in both appetitive and consummatory sexual behaviors reported in this study provided further evidence for the integrative role of POM in male sexual behavior. In addition, the findings of the present chapter indicated that DA in POM is necessary for appetitive and consummatory sexual behaviors. As discussed earlier, the projections of POM that modulates consummatory sexual behaviors are well established (Ball & Balthazart, 2010). On the other hand, the projections of POM that are involved in the motivational aspects sexual behaviors are still not identified (Stolzenberg & Numan, 2011; Wild et al., 2014). The following chapters will address this issue.

Chapter 5 - Effects of Contralateral or Ipsilateral Inactivation of the Preoptic Area and the Ventral Tegmental Area on Male Sexual Behaviors

Rationale

In the previous chapters we found that the mesolimbic system in Japanese quail, similar to its mammalian homologue, is implicated in motivational aspects of sexual behaviors (chapters 2, 3, and 4). In addition, we have discussed and provided evidence suggesting that the POM is involved in the regulation of both appetitive and consummatory aspects of male sexual behavior (chapters 2 and 4). For example, bilateral electrolytic lesions of POM disrupted appetitive sexual behaviors, measured via the learned social proximity response, as well as the male-typical consummatory sexual behavioral responses (Balthazart et al., 1998). If the POM is also modulating motivational aspects of sexual behavior, a regulatory interaction of some sort between the POM and the mesolimbic system is very probable. The possibility that this sort interplay occurs in the regulation of male sexual behavior has been noted by other investigators; however there have been almost no studies that have directly tested this hypothesis (Stolzenberg & Numan, 2011; Will, Hull, & Dominguez, 2014).

On the other hand, several studies have suggested the interactions of these two systems in other motivational domains. For example in rats, mPOA lesions amplified the number of Fos immunopositive cells in nucleus accumbens in response to cocaine administrations (Tobiansky et al., 2013). There have been a number of studies investigating the neural circuits regulating maternal behavior in rats that indicate that there are significant mPOA-mesolimbic system interactions involved in the regulation of these behaviors. For instance, mPOA lesions disrupt pup-induced immediate early gene immunoreactivity (IEG-ir) associated with maternal behavior in Ac (Stack, Balakrishnan, & Numan, 2002). Furthermore, contralateral electrolytic lesions of

mPOA and VTA disrupted the retrieval behavior of mother rats, whereas ipsilateral lesions did not have an effect on this appetitive maternal behavior (Numan & Smith, 1984).

Overall, these findings strongly imply that the interplay between POM and mesolimbic system might also be critical for activation of appetitive sexual behavior. The role of this possible interplay in the regulation of male sexual behaviors has not been investigated to date to our knowledge. To investigate this aim, an asymmetric inactivation procedure had been used: We injected muscimol, a $GABA_A$ agonist, into the POM and VTA either ipsilaterally or contralaterally. This procedure results in a temporary inactivation in the nucleus the receives the injection (reviewed in Edeline et al., 2002). Here we suggest that if the connectivity between these two nuclei and/or their conjunctive effects is critical for appetitive and consummatory sexual behaviors than contralateral inactivation of the two nuclei should disrupt the behavior. In constrast, ipsilateral inactivation should not have a significant effect given the fact that both the POM and the VTA are functional in each one of the two hemispheres.

Materials and Methods

Subjects

The subjects were 20 experimentally naïve adult (~10 weeks old) male Japanese quail (*Coturnix japonica*). In addition, 10 Female Japanese quail had been used as sexual stimuli for behavioral tests and no surgical manipulations were administered. All subjects were maintained on a standard 16L/8D cycle at approximately 22°C and had food and water available *ad libitum*. Male quail were housed in individual cages throughout the experiment, whereas female quail were housed in groups of 5. All of the experimental procedures were in accordance with Johns Hopkins University Animal Care and Use Committee guidelines.

Experimental Procedures

Male Japanese quail were randomly assigned to one of 2 experimental conditions: (1) Ipsilateral or (2) contralateral cannula placement. Before the start of experimental procedures all male quail were tested once, to confirm that they were able to exhibit the full suite of stereotypical sexual behaviors. Subsequently, two permanent guide cannulae were implanted directed at the POM and Ac unilaterally, either on the ipsilateral or contralateral sides. Following one week recovery period appetitive and consummatory sexual behaviors of animals were tested for 4 consecutive days. On day one and three of behavioral testing animal received saline injections whereas on day two and four they received Muscimol injections 30 prior to experimental testing. Brains were collected immediately after the last testing.

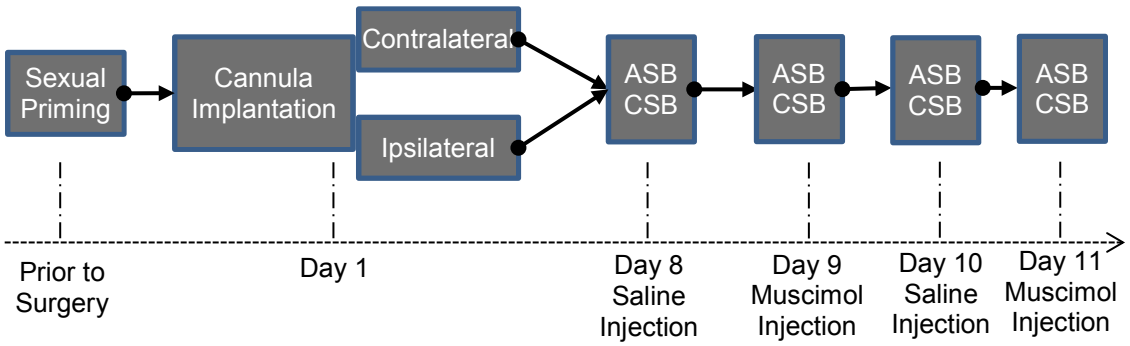

Figure 21. Schematic representation of experimental time line indicating days of appetitive (ASB) and consummatory (CSB) sexual behavior testing, and timeline of surgical procedures

Apparatus and Behavioral Testing

Animals are tested in a large arena to assess appetitive and consummatory aspects of their sexual behaviors (See chapter 4 for details of experimental apparatus and behavioral manipulations). For appetitive sexual behaviors latency of approach and for consummatory

sexual behavior frequency of neck-grabs (NG), mount attempts (MA), mounts (M) and full cloacal contact movements (CCM) had been documented.

Surgical Procedures and Infusions

Prior to surgery quail were first deeply anesthetized with 3-4% isofluorane gas and then placed in a Kopf stereotaxic apparatus. Following the incision a 1/8-in. mounting screw were anchored into the skull. To place the guide acute cannulae (C315GA/SPC, 26 gauge, Plastics One), two holes were drilled for POM and VTA according to the following coordinates: VTA targeted anteroposteriorly (AP) -2.3 mm from ear bars , mediolaterally (ML) ±0.7 from the midline and dorsoventrally (DV) −6 mm from dura. POM targeted anteroposteriorly (AP) + 1.8 mm from ear bars, mediolaterally (ML) ±0.6 from the midline and dorsoventrally (DV) −4 mm from dura. The guide cannula was placed .5 mm dorsal to the POM and VTA to minimize any possible damage to target area. The cannula was fixed in place with acrylic dental cement and fitted with dummy injectors. After these surgical procedures quail were handled daily in the vivarium and the dummy injectors removed and reattached so that the birds would habituate to the experimental manipulations. Infusion of muscimol and saline was administrated via infusion internal cannula (33 gauge, C315IA/SPC, plastic1) which was connected to a plastic tube containing a Hamilton microsyringes in a multiple-syringe pump (KD Scientific). Muscimol (100ng in 0.2 µl) or 0.9% saline solution (0.2 µl) was infused to POM and VTA over a 2 minute period. For the last administration of a fluorescent conjugated muscimol (M-23400, Life technologies) was used so that we would be able to assess the location and spread of the infusion. In contrast, the initial administration was done with a solution of muscimol at the same

concentration but with no fluorescent tags (CAS 506044, Millipore). Subsequent to termination of the infusion the infusion cannula remained in the site for the following 2 minutes. All behavioral studies were performed 20-30 minutes after the infusion. Previous studies have suggested that effects of muscimol are present as soon as 10 minutes after the administration and persisted for several hours (Krupa, Ghazanfar, & Nicolelis, 1999) . Five of the Japanese quail, 3 contralateral and 2 ipsilateral, showed correct cannulae placements in both VTA and POM, other animals excluded from all statistical analysis. Even though cannulae placements were within POM, fluorescence also documented surrounding POA.

Results

Behavioral Data

The histogram represent the mean latency of approach and mean number of mounts and mount attempts subsequent to contralateral or ipsilateral infusions of muscimol (testing days 2 and 4) and saline (testing days 1 and 3) (Figure 26). A repeated measures ANOVA indicated a significant difference on test sessions (muscimol or saline) ($F(1, 3) = 11.33$, $p<.05$). In addition, an interaction between test session and ipsilateral-contralateral placement of cannula was also present ($F(1, 3) = 11.05$, $p<.05$) for latency approach. On the other hand no significant difference was present for test session ($F(1, 3) = .28$, $p>.05$) or test session- ipsilateral-contralateral placement interaction ($F(1, 3) = .05$, $p>.05$).

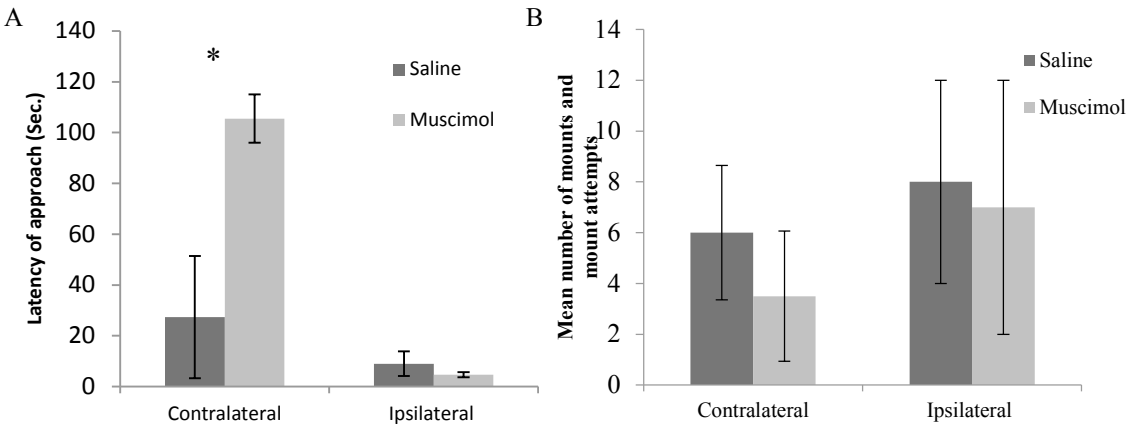

Figure 22. Appetitive and consummatory sexual behaviors of contralateral or ipsilateral infusions of muscimol and saline treated animals: Panel A demonstrates the latency of first sexual approach within 2 min quantification period to assess appetitive sexual behaviors/ Panel B represents the frequency of mount attempts and mounts within 2 min quantification period to assess consummatory sexual behaviors. Error bars represent the standard error of the mean.

Discussion

The results of the current study provide novel evidence for interplay between POM and VTA in modulation of appetitive but not consummatory sexual behaviors. There is a reasonably large body of evidence that has implicated the POM in the regulation of both appetitive and consummatory aspects of sexual behavior in male quail (Balthazart et al., 1998; Cornil et al., 2015; Iyilikci et al., 2014; Taziaux et al., 2006; Tlemçani et al., 2000) . In contrast the mesolimbic system has been linked predominantly to the regulation of appetitive aspects these behaviors. It is useful to note that POM-VTA projections have been implicated in the regulations of other neural systems that control motivated behaviors (reviewed in Stolzenberg & Numan, 2011). Based on this evidence we suggest that POM is modulating the mesolimbic system when it facilitates the initiation of goal-directed appetitive behaviors via its projections to VTA. However, asymmetrical inactivation studies do not provide information on the nature of interaction between the two nuclei. There are several possible ways this interaction may occur

for example: (1) Projections from POM-VTA or VTA-POM might be directly driving this effect, or (2) the cumulative effect of these two nuclei may influence other regions.

Muscimol is a $GABA_A$-agonist, and can suppress neural activity with a short latency in the area of infusion (Allen et al., 2008). A number of studies have examined muscimol mediated inactivation of the mPOA based on measurements of a variety behavioral tasks (e.g. Arrati et al., 2006; Hunt, Zaretsky, & Sarkar, 2010; Rusyniak, Zaretsky, & Zaretskaia, 2011). However, there are conflicting results for VTA: Some studies reported that a muscimol infusion in VTA facilitates the firing rates of dopaminergic neurons (Xiao, Zhou, Li, & Ye, 2007), resulting in an increase in dopamine release in Ac (Xi & Stein, 1998), whereas other studies utilizing similar manipulations have reported a decrease the dopamine release in Ac (Westerink, Kwint & deVries 1996). These contradictory findings have also been observed for the behavioral studies; some studies have documented that muscimol in the VTA is associated with the augmentation of the behavior of interest whereas others documented an inhibition (Munro & Kokkinidis, 1997; Numan et al., 2009). Numan et al., (2009) suggested that the disruption in the maternal behaviors subsequent to muscimol injections might be due to hyperactivation of dopaminergic neurons in VTA. The effects of muscimol in avian VTA has not been studied, therefore we do not have the evidence to argue if the observed change in the behavior is due to hypo- or hyper- activation of dopaminergic neurons. However, the dissociations we observed between the contralateral and ipsilateral injection strategies on appetitive and consummatory behaviors are quite consistent with the hypothesis that the POM and VTA are functionally interacting in regulation of appetitive sexual behaviors. However, it remains an open question as to how exactly the interplay between POM and VTA exerts their effects on appetitive sexual behaviors. One possible way might be through projections from POM to VTA. Thus in the following chapter we

will confirm the connections between POM and VTA using retrograde tracing and combine this with the expression of immediate early genes to test if these connections have functional significance.

Chapter 6 - Sociosexual Interactions Induce Fos Immunoreactivity in Hypothalamic Cell Groups That Project to Ventral Tegmental Area

Rationale

In the previous chapters, we have presented evidence implicating the involvement of the POM and mesolimbic systems in the regulation of appetitive sexual behaviors (e.g., Balfour et al., 2004; Iyilikci et al., 2014; Taziaux et al., 2006). In addition, inactivation data from the previous chapter imply that the POM and VTA interaction is critical in regulation of appetitive sexual behaviors. However, the nature of this interaction is still not well understood; this chapter aims to explore one of the possible ways of this interplay.

In rats, projections from mPOA to VTA have been documented via tract tracing methods (Simerly & Swanson, 1988; Tobiansky et al., 2013). Although limited, there are some studies suggesting that projections originating from mPOA to VTA are functionally involved in the regulation of other motivated behaviors, particularly in appetitive maternal behaviors, a number of studies have investigated this possibility (reviewed in Stolzenberg & Numan, 2011).

For instance, Fos-ir within POM and Ac is elevated in response to maternal behaviors, and a subset of these cells in POM project to the VTA, indicating that these projections are associated with appetitive maternal behaviors in female rats (Numan & Numan, 1997). In addition, mPOA lesions disrupt pup-induced immediate early gene immunoreactivity (IEG-ir) associated with maternal behavior in Ac (Stack, Balakrishnan, & Numan, 2002). Furthermore, contralateral electrolytic lesions of mPOA and VTA disrupted the retrieval behavior of mother rats, whereas ipsilateral lesions did not have an effect on this appetitive maternal behavior (Numan & Smith, 1984).

Overall, these studies suggest that mPOA-VTA projections facilitate appetitive sexual behaviors on rat maternal behavior. On the other hand, interestingly, mPOA lesions increased cocaine-induced Fos-ir in the nucleus accumbens and conditioned place preference in female rats (Tobiansky et al., 2013). This study also demonstrated that the 67% of mPOA-VTA efferents were GABAergic. Together with behavioral and Fos findings, this study suggests that mPOA-VTA projections may also exert inhibitory inputs onto the mesolimbic system.

These studies indicate that mPOA-VTA connections may have a significant role in the control of appetitive behaviors in a general context. However, this modulation may exert its effects through direct and/or indirect projections. The two most likely nuclei that may mediate this POM-VTA circuit are the (1) paraventricular nucleus of the hypothalamus (PVN) and (2) lateral hypothalamus (LHA).

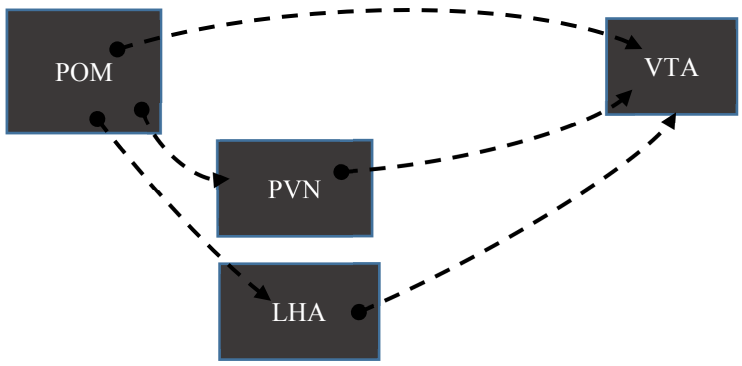

Figure 23. A schematic representation of putative projections of POM in control of male sexual behavior.

Paraventricular Nucleus of the Hypothalamus

Anatomical studies have demonstrated that PVN has reciprocal connections to POM and VTA (Korf, 1984) . PVN lesions in rodents impair male sexual behaviors (Liu, Salamone, & Sachs, 1997). In addition, a subset of oxytocinergic neurons in PVN are known to project to VTA (Rutherford, Williams, & Moy, 2011). Indeed, oxytocin injections into PVN induce penile erections and stimulate DA release into Ac in rats (Melis, Argiolas, & Gessa, 1989; Melis et al., 2007; Sanna et al., 2007).

Critically, avian species do not retain the mammalian peptide oxytocin or vasopressin; instead, they have a diverse oligopeptide, vasotocin, which is a homologue of both mammalian oxytocin and vasopressin (Goodson & Bass, 2001) . Similar to its mammalian homologue, vasotocin neurons are also implicated in sexual behaviors. For example, an increase in the number of vasotocin-containing cells has been observed during the breeding season in Japanese quail (Singh & Chaturvedi, 2008). In addition, an elevated FOS immunoreactivity has been observed in vasotocin cells in chickens subsequent to sociosexual interactions (Xie, Kuenzel, & Sharp, 2011) . These studies suggests that POM-PVN-VTA connections are a candidate pathway for the interaction of these two systems.

Lateral Hypothalamus

Another possible pathway for the hypothalamic interaction to mesolimbic circuitry might be through the POM-LHA-VTA pathway. The hypocretinergic (orexinergic) neurons in the lateral hypothalamus have been associated with a number of motivated behaviors, such as food intake and drug-seeking behaviors (Boutrel, Kenny, & Specio, 2005; Kotz, 2006; Sakurai, Amemiya, Ishii, & Matsuzaki, 1998; Sebastiano & Coolen, 2011). Currently, accumulating

evidence suggests that these neurons might be implicated in male sexual behavior as well: infusions of hypocretin to the hypothalamus facilitates sexual behavior in male rats (Gulia, Mallick, & Kumar, 2003), hypocretinergic receptor blockade impairs consummatory sexual behaviors, and also an elevated Fos – hypocretin double-label immunoreactivity has been documented subsequent to the sexual behaviors in the lateral hypothalamus of the male rats (Muschamp et al., 2007). In addition, these hypocretinergic (orexinergic) neurons have bidirectional connections with mPOA and VTA (Sakurai, Nagata, Yamanaka, & Kawamura, 2005). More interestingly, injections of hcrt-1 (a hypocretin neuropeptide precursor) into the VTA increased firing rates of the dopaminergic neurons within the VTA in a dose-dependent manner (Muschamp et al., 2007). Overall, these studies provide ample evidence that hypocretinergic (orexinergic) neurons within the LHA are implicated in sexual behavior, and that the LHA projections to the VTA may play a role in modulating sexual behavior.

Based on these findings, we aimed to investigate these three potential pathways that are likely implicated in sexual behavior: ((1) Direct projections from POM to VTA, indirect projections through (2) PVN and/or (3) LHA). One way to assess these possibilities is by using neural tract-tracing methods in combination with immediate early genes. To this aim, we injected biotinylated dextran amine (BDA, 3kDa) into ventral tegmental area. At this molecular weight (3kDa), BDA retrogradely labels cell bodies that send projections to the injection site (Reiner et al., 2000). By using double-label immunohistochemistry for Fos and BDA, we examined the cells within POM, PVN, and LHA that projected to VTA and responsive to sexual behaviors (See figure 23).

Materials and Methods

Subjects

Twelve adult (10 weeks old) male and 6 female quail were used in the present study. Japanese quail were obtained from a local breeder (Maryland Exotic Birds). All subjects were housed individually and maintained on a standard 16L:8D cycle to insure that they are reproductively active (Ball & Balthazart, 2010). All animals had food and water available *ad libitum*. Male quail were housed in individual cages throughout the experiment, whereas female quail that are used as stimuli were housed in groups of 5. All of the experimental procedures were in accordance with Johns Hopkins University Animal Care and Use Committee guidelines.

Experimental Procedures

Male quail were randomly assigned to two groups: Sexual behavior (SB), and control group (n=6 per group) that remained the same throughout the experiment. At the start, all subjects experienced a 15 minute pretest in which males and females interacted which ensured males were sexual active. Subsequently, biotinylated dextran amine (BDA, 3kDa) was bilaterally injected into the ventral tegmental area. 15 days after the BDA injections, animals in the sexual behavior group were placed in to the experimental chamber with a sexual active female where they engaged in stereotypical appetitive and consummatory sexual behaviors, whereas animals in the control group were placed in the holding cage individually. All animals returned to their home cages after the 15 minute manipulation and remained in the home cages for the next 75 minutes until their brains were collected (see figure 24).

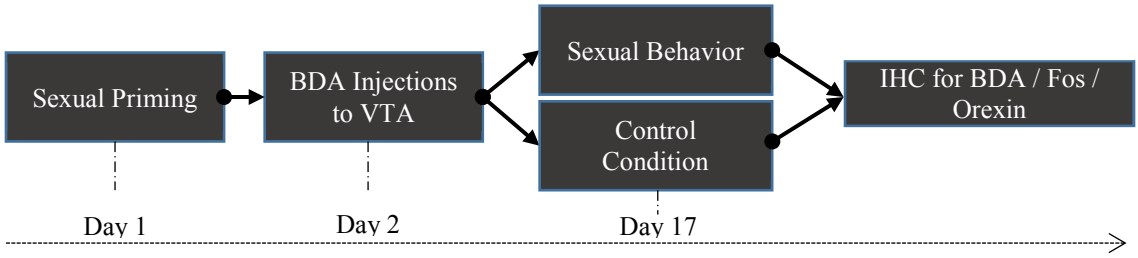

Figure 24. A schematic representation of experimental time line; indicating pretesting for gaining sexual experience, stereotaxic injection of retrograde tracer BDA and experimental manipulations.

Retrograde Pathway Tracing

For neuronal tracing adult male Japanese quail were anesthetized using isoflurane and placed in a stereotaxic apparatus. Biotinylated dextran amine (BDA, 3kDa) was stereotaxically injected into the putative Ac as a retrograde neuroanatomical tracer. At this molecular weight (3kDa), BDA retrogradely labels cell bodies that send projections to the injection site (Reiner et al., 2000). The skull was open above the target brain region and a Hamilton Neurosyringe was lowered to the desired coordinates, AP:-2.3, ML±.7, DV:-6 . The zero coordinate for AP was taken from ear bars. Upon reaching the desired dorsal-ventral coordinate, 250 nanoliters of BDA was injected using pressure injection. The syringe remained in the brain for 5 minutes and was removed slowly. The skin was sutured, birds recovered under a heat lamp, and were then returned to their home cage for 15 days; and then their brains were extracted as described above.

The stereotaxic BDA injections of 4 quail were not released into VTA so they are excluded from all further analysis. For the remaining 7 animals that had bilateral VTA injections, BDA positive perikarya was present in POM, PVN, and LHA.

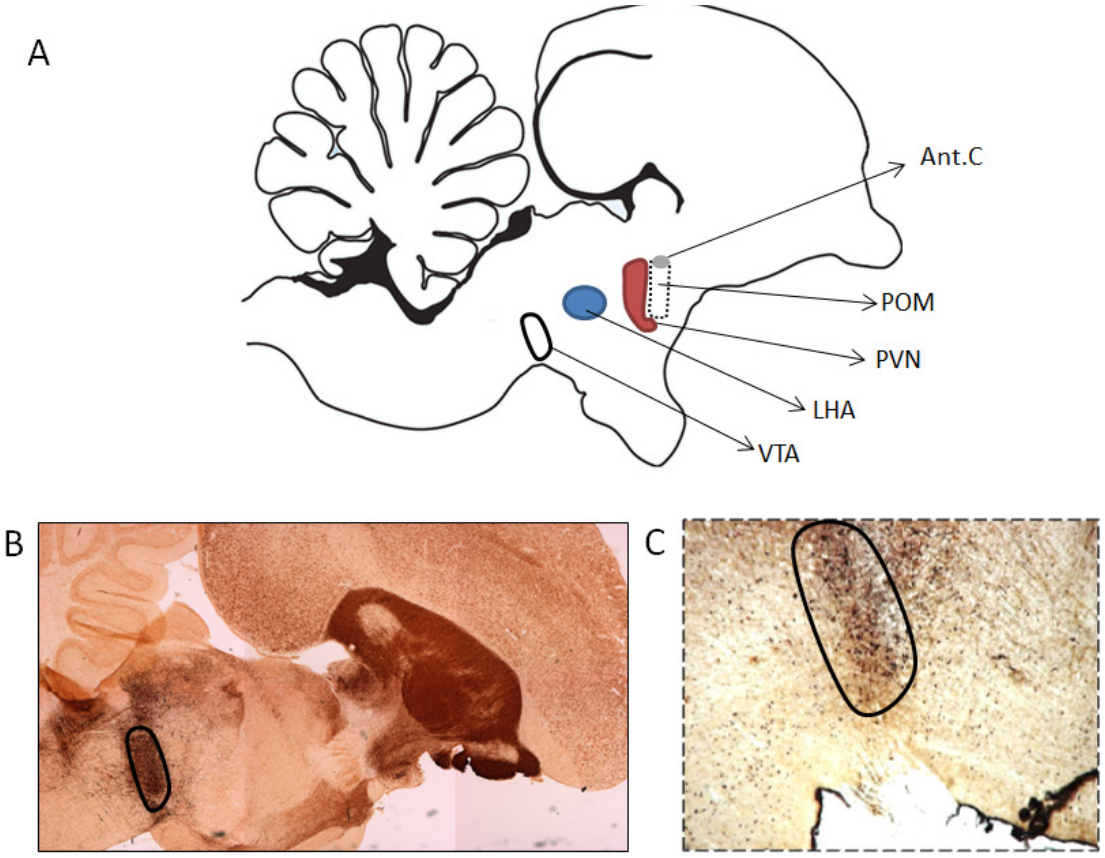

Figure 25. Panel A shows the schematic drawings of sagittal sections of the quail brain illustrating the areas where the immunopositive cells quantified. In panel B and C photomicrographs illustrating the ventral tegmental area by (B) TH immunohistochemistry of quail brain for identifications of nucleus in sagittal sections, and (B) an example of BDA injection into VTA.

Fixation and Immunohistochemistry

Ninety minutes following onset of the behavioral tests, subjects were decapitated and their brain dissected out of the skull. The brains were placed into acrolein (5% in phosphate buffer 0.1 M saline) for 3 hours, washed four times in PBS (15 min) and cryoprotected in 30% sucrose for 24 h at 4 °C. The brains were then frozen on dry ice and stored at −70 °C until used. All brains were cut at 40 μm in the coronal plane using a cryostat at –20°C and sections were collected in four series.

BDA was visualized using a standard avidin-biotin horseradish peroxidase (ABC) staining procedure on free-floating, acrolein-fixed quail tissue. Tissue was transferred to PBS and washed for 5 minutes three separate times and then washed in 0.1% sodium borohydride for 30 minutes. After three more 5-minute PBS washes, tissue was incubated for 30 minutes in 3% hydrogen peroxide to block any endogenous peroxidase activity. After three PBS washes, tissue was incubated for 4 hours in ABC. Tissue was washed in PBS and then immersed in sodium acetate for 5 minutes. Tissue was then exposed to diaminobenzidine (DAB). The reaction was stopped with sodium acetate, and tissue was washed in PBS.

Subsequently, Fos expression was then also visualized by immunohistochemical procedures with the ABC technique on the same tissue. Three rinses with 0.01 M PBS containing 0.1% Triton X-100 were performed between each step. First, sections were incubated for 60 min in 0.3% hydrogen peroxide and in 20% normal donkey serum to remove endogenous peroxidase and decrease non-specific binding. This step was followed by Avidin-biotin blocking (Vector SP-2001) for 15 min, to block possible biotin binding sites in the tissue. Then sections were incubated in the primary Fos antibody (1:10,000) for 48 hours. Afterwards, sections were incubated for 60 min in donkey anti-rabbit serum. The antibody-antigen complex was localized using the avidin-biotin complex method performed with a Vector Elite Kit (ABC Vectastain Elite PK-6100, Vector Laboratories PLC) and finally, the peroxidase enzymatic activity was visualized with DAB (3,3'diaminobenzidine tetrahydrochloride) intensified with Nickel ammonium sulfate and chloride to have a black color.

A similar technique was used for immunohistochemical labeling of Orexin in series of sections that were already labeled for Fos and BDA with the following exceptions. Sections were incubated in Orexin antibody (1:5,000, Santa Cruz, sc-8070) for 48 hours. Afterwards, sections

were incubated for 60 min in biotinylated horse anti-goat IgL. The peroxidase enzymatic activity was visualized with a HRP substrate to produce blue-gray reaction (Immpact-SG Peroxidase, Vector, SK-4705). Reactions were terminated by several rinses in PBS. Then sections were mounted on gelatin-coated slides. Slides were coverslipped using exposure to successively higher concentrations of ethanol and then exposure to xylene and coverslipped using permount.

Quantification of all BDA-ir, Fos-ir, Orexin-ir including double labelled and triple labelled cells was done by an experimentally blind observer under a light microscope by direct observation. To validate these counts a different experimentally blind observer collected data a high correlation between the observers was present ($r(91)=.908$, $p<.05$).

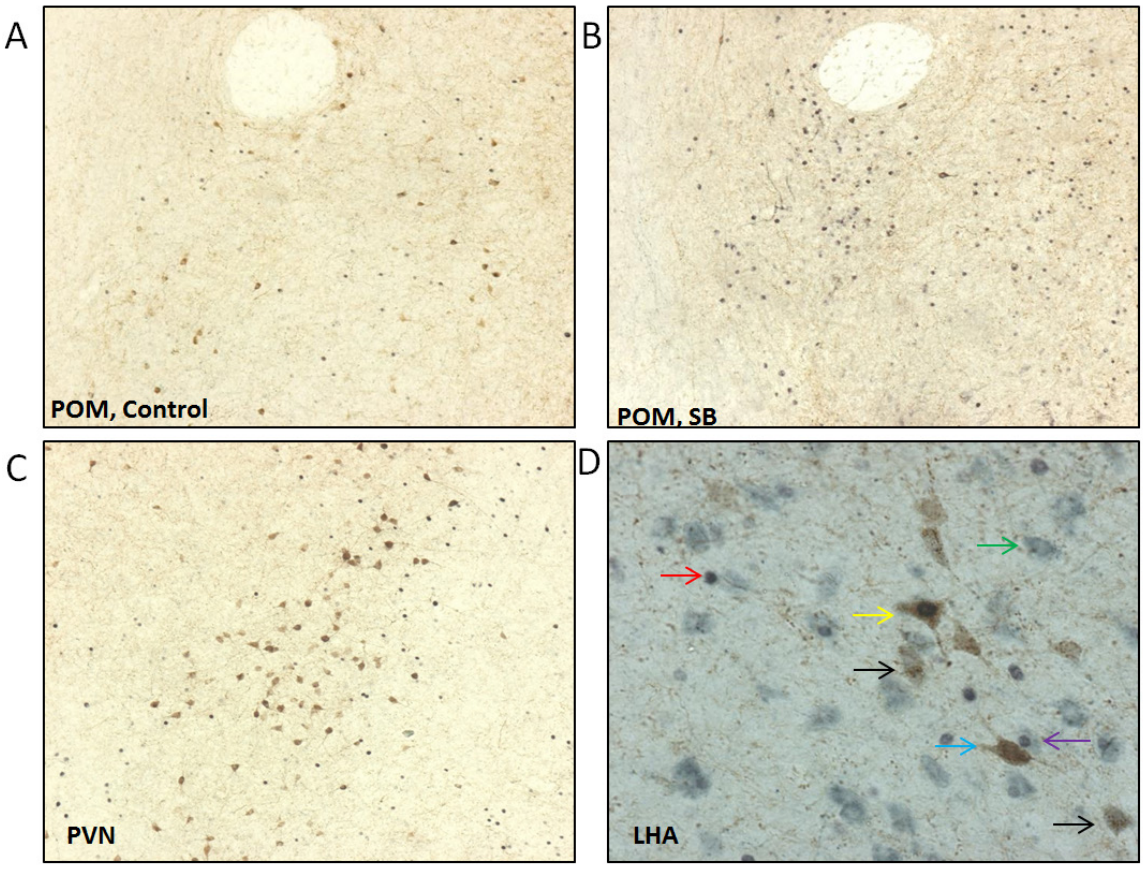

Figure 26. Photomicrographs illustrating the Fos, BDA and orexin-ir in sagittal sections of Japanese quail brain.

Photomicrographs showing the sagittal section of POM of male Japanese quail in (A) control and (B) sexual behavior condition. Panel C illustrates Fos and BDA labelled cells in the PVN. Panel D illustrates single Fos-ir positive (red arrow), single orexin (green arrow), single BDA (blue arrow), double labelled BDA and Fos (yellow arrow), Double labeled orexin and Fos (purple arrow), and also double labelled orexin and BDA (black arrows).

Results

Figure 27 presents the mean number of Fos-ir and BDA+Fos-ir double labeled cells within POM. Independent sample t-tests yielded significant differences for both Fos-ir ($t(5)=3.47$, $p<.05$) and percentage of BDA+Fos-ir in BDA-ir cells ($t(5)=3.79$, $p<.05$) cell numbers.

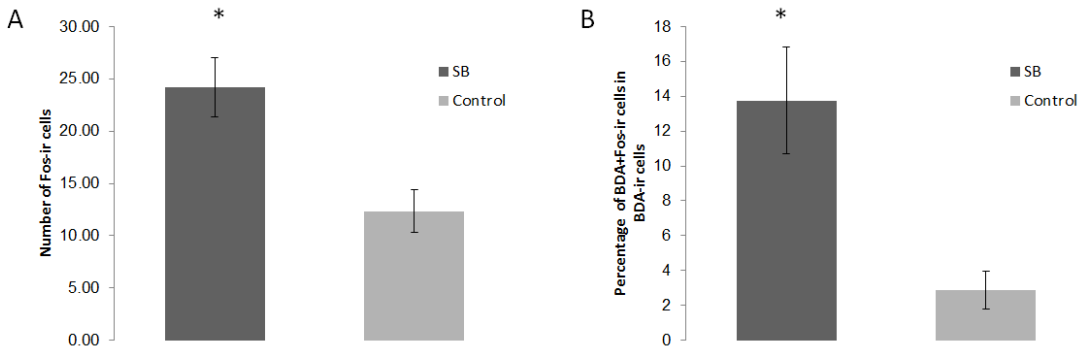

Figure 27. Mean number of (A) Fos-ir cells and (D) BDA and Fos double labelled cells in the POM of experimental and control groups. Error bars represent the standard error of the mean.

Independent sample t-tests demonstrated no significant differences in Fos-ir ($t(5)=2.12$, $p>.05$) or percentage of BDA+Fos-ir in BDA-ir cells ($t(5)=1.91$, $p>.05$) between sexual behavior and control groups in PVN (see figure 28 A & B).

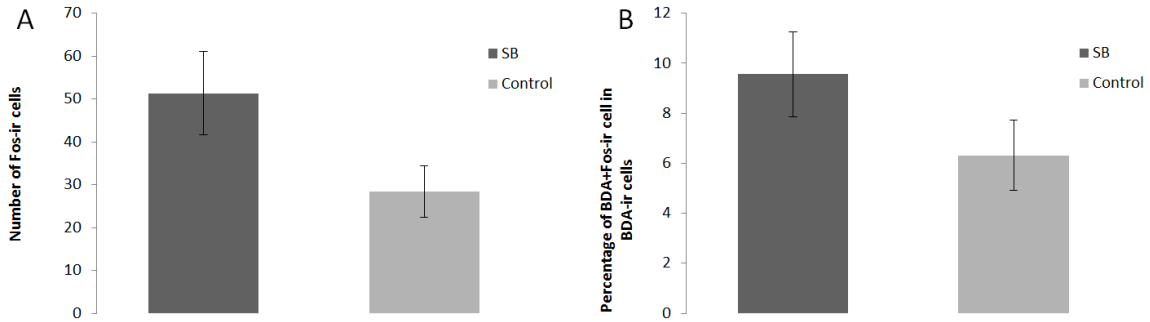

Figure 28. Mean number of (A) Fos-ir cells and (D) BDA and Fos double labelled cells in the PVN of experimental and control groups. Error bars represent the standard error of the mean.

The independent sample t-tests indicated no significant group difference in the numbers of Fos immunopositive cells within the lateral hypothalamus (LHA) ($t(5)=1.94$, $p > .05$). The corresponding analysis found no significant difference in the percentage of Orexin-Fos-ir cells within the Orexin-ir cells ($t(5)=1.86$, $p > .05$). The independent t-tests comparing the percentage of BDA-Fos positive cells within BDA cells of LHA indicated a significant difference ($t(5)=2.66$, $p < .05$).

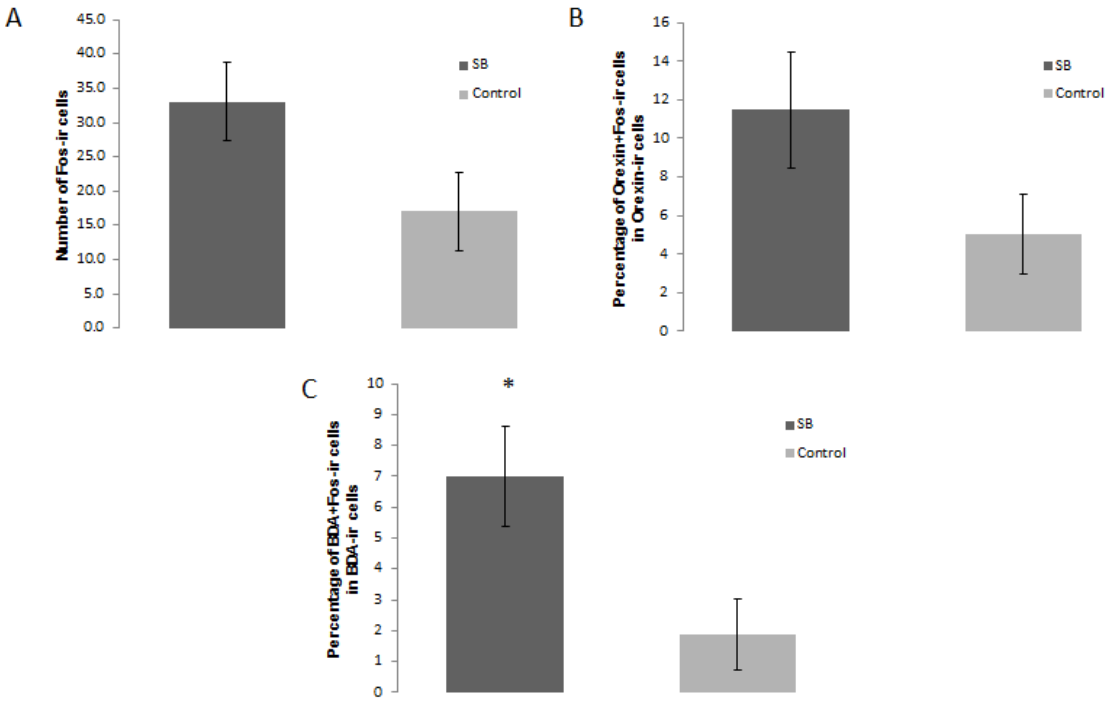

Figure 29. Mean number of (A) Fos-ir, (B) orexin-Fos double, and (C) BDA-fos double labelled cells in the LHA of experimental and control groups. Error bars represent the standard error of the mean.

Discussion

The present chapter revealed an enhanced Fos immunoreactivity in POM subsequent to expression of sexual behaviors, this effect was not observed in the PVN or LHA. In addition, we found an increase in BDA and Fos double-labelled immunopositive cells within the POM and LHA but not in the PVN. In addition, in order to differentiate the lateral hypothalamus anatomically, orexinergic cells were identified; thus we also explore the Orexin and Fos double-labelled cells. Despite the fact that a big numerical increase documented for the mean number of Orexin and Fos double labelled cells in sexual behavior group no significance differences were present. We also detected a limited number of Orexin-BDA-Fos triple labelled cells, but since the frequency of these were low and they were not present in every animal no statistical analysis were performed.

Fos Induction in POM

The significant role of POM in modulation of sexual behaviors have been disccused numerous times in previous chapters, and in concordance with earlier studies we found a significant increase in Fos-ir associated to sexual behaviors (Charlier et al., 2005; Hamson & Watson, 2004; Heeb & Yahr, 1996; Iyilikci et al., 2014; Portillo & Paredes, 2004; Robertson et al., 1991; Wersinger & Baum, 1997). Also, in the previous chapter, we demonstrated that connectiontivety between POM and VTA or their cummulative intpus is implicated in sexual behaviors. This, study allowed us to explore one of the possible ways that this interplay may manifest itself. First, via retrograde tract tracing we demonstrated that POM projects to VTA in Japanese quail. The mean number of BDA immuno positive cells in POM was 31.4 for the sum of samples collected for each animal. More interestingly, this study showed that a significantly

higer percentage cells that are projection to VTA were Fos immunopositive, providing an evidence for functional significance of POM-VTA projections in regulation of sexual behaviors.

Fos Induction in PVN

PVN's hodology in relation to POM and VTA (Korf, 1984), and its involvement in sexual behaviors in rats (Liu, Salamone, & Sachs, 1997), and in avian species (Singh & Chaturvedi, 2008; Xie, Kuenzel, & Sharp, 2011) led us to explore it as a mediatory nucleus in POM, mesolimbic system interactions. However, we found no significant increases either in Fos-ir or in BDA-Fos double labeled cells.

Fos Induction in LHA

LHA and orexinergic cells in LHA are also documented to regulate reward related behaviors (Harris, Wimmer, & Aston-Jones, 2005; Tsujino & Sakurai, 2009), including male sexual behavior (Gulia, Mallick, & Kumar, 2003; Muschamp et al., 2007). In addition hypocretinergic (orexinergic) neurons have bidirectional connections with mPOA and VTA (Sakurai, Nagata, Yamanaka, & Kawamura, 2005). Here, we were not able to demonstrate that sexual behavior induced Fos immunoreactivity in in LHA, or in the orexinergic cells of LHA. However, within the cells that are projecting to VTA an increase in Fos-ir was detected.

Together, these data support the view that projections from POM to VTA have a functional significance in the regulation of sexual behaviors. We also demonstrated that projections originating in LHA are also implicated in sexual behaviors in Japanese quail.

However, this study did not explore the possible POM-LHA connections, so we do not have evidence to conclude if the part of the modulatory effect of POM on VTA is through LHA.

Chapter 7 - General Discussion

A major goal of this dissertation is to elucidate if POM-mesolimbic system interactions are involved in motivational aspects of sexual behavior, and if this interplay is in part through functional connectivity between POM and VTA.

The first experiment (Chapter 2; Iyilikci et al., 2014) employed immediate early gene expression techniques to demonstrate that different monoaminergic cell groups are specifically activated, in a sex specific manner, in relation to different aspects of sexual behavior. The findings from this study strengthen the view that the POM plays a critical role in the regulation of male sexual behavior including both appetitive and consummatory component of these sexual behaviors. Behavioral studies (see Beach 1956, and Everitt 1990 for reviews) suggested that appetitive and consummatory aspects of male-typical sexual behavior could be differentially controlled by various aspects of the integrated hypothalamic-limbic circuit that controls sexual performance and motivation. Our findings indicate that there are differences in the control of these different aspects of male sexual behavior within the POM itself which is consistent with previous findings based on selective lesions (Balthazart et al., 1998 Journal of Neuroscience). Previous evidence strongly suggests that BSTM, VTA and PAG are also components of the circuit regulating male-typical sexual behaviors and that the data obtained here on the total number of Fos-ir cells in BSTM, VTA and PAG is also in concordance with these findings (reviewed in Ball & Balthazart 2010; Dominguez & Hull, 2005). VTA is a critical part of the mesolimbic reward circuitry, and had been associated with the regulation of sexual behavior in mammals (Will et al., 2014), in particular its projections to nucleus accumbens have been implicated in the regulation of a number of motivated behaviors (Kelley & Berridge, 2002). However, we were not able to report any results concerning Ac in this first study due to the

reported inconsistencies in the anatomical location of Ac in avian species, which had now been addressed in subsequent studies completed as a part of this dissertation.

A novel feature of this first study involves the investigation of possible modulatory action by the serotonergic system, which has not been subject to investigation in avian species. Cells in the Raphe pallidus did not exhibit Fos expression in association with appetitive behaviors but did in association with consummatory behaviors in males. The number of TPH cells expressing Fos was only elevated when males engaged in copulatory behavior per se. The inhibitory role of serotonin in sexual behavior is well established in rodents (Fernandez-Guasti et al., 1992; Lorrain et al., 1997). For instance, an increase in 5-HT concentrations has been documented in the lateral hypothalamic area after ejaculation (Lorrain et al., 1997). The present results are thus consistent with the notion that serotonergic inputs rooted in the raphe pallidus may be responsible for the modulation of the satiation of male sexual behavior for male Japanese quail. Overall this study provided valuable evidence concerning the roles of different monoaminergic neurotransmitters in modulation of sexual behavior. However, it also pointed to two major limitations: First of all, although there are a number of ways to test male appetitive behavior (Adkins-Regan & Leung, 2006; Domjan & Hall, 1986), there has been a paucity of behavioral paradigms that address the analogous behaviors in female quail, a trend that have also been observed in other animal models and behavioral paradigms (Beery & Zucker, 2011; Clayton & Collins, 2014). In addition, due to controversies on the exact anatomical location of the nucleus accumbens (Ac) we were unable to report any results from this critical part of the mesolimbic system.

Accordingly, the next series of experiments addressed these issues (Chapter 3). First we delineated the borders of Ac via different immunohistochemical markers that are known to mark

the boundaries and sub-nuclei of Ac in mammalian species. For instance DARPP-32 is strongly expressed in mammalian Ac (Perez & Lewis, 1992; Schalling et al., 1990), here we documented similar results and this marker enabled us to delineate the borders for putative Ac and BSTl. However no specific differentiation was observed between Ac and Mst. Markers of the serotonin transporter was also densely present in the mammalian Ac (Brown & Molliver, 2000). Our investigations in Japanese quail, demonstrated that the distribution pattern of serotonin transporter is very useful tool to differentiate Ac; this marker enabled us to delineate the borders of presumptive Ac and the two neighboring brain areas, the Mst and BSTl. In addition, we explored if there is a core-shell distinction in avian Ac as has been described in its mammalian homologue. In mammals, a relative difference has been documented in the distribution of calcium-binding proteins in the core and shell region of the Ac (Brauer et al., 2000; Bubser et al., 2000; Hartig et al., 2003; Tan et al., 1999). In Japanese quail we documented relatively more expression in the putative shell region of the Ac for a calcium-binding protein calretinin. To further investigate the homologies between mammalian and avian Ac we injected a retrograde tracer, BDA, into the region defined as Ac that has been suggested by our immunohistochemical markers. Similar to its mammalian homologue, Ac of quail receives projections from VTA, Hp, BSTl, Rp, MVcp and MDcp (Delfs, Zhu, Druhan, & Aston-Jones, 1998; French & Totterdell, 2002). Importantly, injections made outside of the Ac in the surrounding Mst demonstrated different retrograde labeling patterns. These two lines of evidence indicated that the hodological, immunohistochemical markers of the putative Ac in Japanese quail are comparable to its mammalian homologue. Consequently we decided to test if the nucleus is also functionally similar; therefore we measured the pattern of immunoreactivity of immediate early gene Egr-1- in the Ac of male Japanese quail and documented a significant enhancement of Egr-1 in response

to socio-sexual interactions. A similar increase in the number of IEG immunopositive cells in Ac in mammals has also been documented (Balfour, Yu, & Coolen, 2004). We also wanted to test if the Ac is involved in female sexual motivation. However, as previously mentioned, there are a paucity of behavioral tests available that assess female appetitive behaviors in quail and other birds. It was previously reported that females based on phonotaxis measures find male conspecific vocalizations, i.e., crows, sexually attractive (Goodson & Adkins-Regan, 1997). Based on this premise we investigated the approach behavior of females. We documented that a majority of the females that were exposed to conspecific male vocalizations exhibited a positive phonotaxic response towards the speakers, whereas, females in the control condition, which were presented with reversed playbacks of the same vocalizations did not show these behaviors. We categorized this approach behavior as an example of appetitive sexual behavior. Furthermore, the documented increase in Egr-1 immunoreactivity in Ac of the females that approach the speaker, relative to the animals exposed to reverse playbacks of the crows, supports our interpretation of this behavior. Taken together, these histochemical, hodological, and functional findings help to elucidate the anatomical location of the avian Ac and its subdivisions. Previously, it has been argued that brain regions involved in critical adaptive decisions that are linked to social behaviors along with the underlying reward systems are evolutionally conserved in all vertebrates (O'Connell & Hofmann, 2012). This idea was criticized on the basis of insufficient data on anatomical homologies of the mesolimbic system among vertebrates and its involvement in social behaviors (Goodson & Kingsbury, 2013) . The reported experiments provide additional evidence that the Ac in birds and mammals is evolutionarily conserved, and implicated in socio-sexual behaviors.

After establishing anatomical location for the Ac in Japanese quail, in the next series of experiments we aimed to investigate how Ac might be involved in regulating sexual behaviors. As described earlier, the dopaminergic inputs to Ac from VTA have been implicated in the process of attributing incentive salience to rewarding stimuli (Berridge & Robinson, 1998; Kelley & Berridge, 2002). In addition, a previous study documented an increase in the extracellular dopamine in POM of male Japanese quail when a conspecific female is presented suggested that dopaminergic inputs to POM may also be implicated in appetative sexual behaviors (Kleitz-Nelson, Dominguez, & Ball, 2010). Thus to investigate the specific role of dopamine in POM and Ac we injected 6-OHDA to deplete the dopaminergic inputs these two nuclie. Animals exposed to 6-OHDA exhibited a rapid impairment in both aspects of sexual behavior and this impairment persisted for 5 hr and 24hr after 6-OHDA injections in both POM and Ac compared to sham injections. However, there was complete recovery of these behaviors 1 week after surgery. Overall, these data are consistent with the notion that dopamine action in the Ac and POM is needed to activate sexual motivation.

Along with the previous findings, the data in the above mentioned chapters' established and anatomical location for the avian Ac, and demonstrated that the mesolimbic system, VTA and Ac, is implicated in the regulation of sexual motivation in Japanese quail. In addition, we provided further evidence that POM is necessary for both appetitive and consummatory sexual behaviors. On top these findings; there is large body of knowledge indicating that POM is a major site of hormone actions that controls both appetitive and consummatory sexual behaviors. For example, testosterone implants targeted specifically to POM restore the sexual behaviors subsequent to castrations, furthermore aromatase inhibitors in POM impairs all these behaviors (Balthazart & Surlemont, 1990). If the motivational state of an animal is linked to its hormonal

milieu, and again if the POM is necessary and sufficient for hormones to exert their effects on sexual behavior than there should be an interplay between POM and mesolimbic system in regulation of motivational aspects of sexual behaviors. The possibility of this interplay has been noted by other researchers; however there have been no studies directly testing this hypothesis in a clear manner for the case of male sexual behavior (Stolzenberg & Numan, 2011; Will, Hull, & Dominguez, 2014). To test this idea, asymmetric inactivation procedures were employed in studies described in chapter 5. We documented that ipsilateral `inactivation did not have an effect on appetitive or consummatory sexual behaviors. On the other hand, contralateral inactivation of POM and Ac specifically impaired appetitive sexual behaviors. The disassociation between the two aspects of male-typical sexual behavior in the contralateral inactivation condition suggests that POM-VTA interactions are critical in the modulation of motivational aspects of sexual behaviors. Even though this study provides evidence for the existence of this interplay it does not establish how this interplay may occur. To investigate one of the possible pathways that may underlie this interplay, in the last set of experiments we investigated afferent projections to ventral tegmental area. We documented an increase in Fos immunopositive cells in POM and LHA that are projecting to VTA in response to sexual behaviors. Along with the previous reported experiments, these data are consistent with the view that projections from POM to VTA have a functional significance in the regulation of sexual behaviors.

A Model for Appetitive and Consummatory Sexual Behaviors

What have the studies reported in this dissertation told us that is new about our understanding of the neural control of sexual behavior? The POM is a key integrative site in the

male sexual behavior circuit in quail (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013) as is the case in other vertebrate species (Hull, 2011). Although there are many gaps in our knowledge concerning its hodology, this brain area seems to receive the relevant sensory inputs in relation to sexual stimuli. In addition, the POM is responsive to the endogenous hormonal milieu and thus to the environmental conditions that might modulate hormone release. Japanese quail tend to breed seasonally during the spring and summer season (Robinson & Follett, 1982). As is the case for many other species that exhibit this pattern of reproductive behaviors, this is a photoperiodic species (Follett & Sharp, 1969): in the case of male quail increases in day-length cause an increase in the size of the cloacal gland (Oishi & Konishi 1983), testes (Follett, 1976) and the size of the POM (Panzica, Viglietti-Panzica, & Balthazart, 1996; Thompson & Adkins-Regan, 1994). Therefore hormonal actions in POM potentially enable the animal to adjust its sexual behavior, including the associated goal-directed behaviors, to the particular physical and developmental conditions. For example, administering aromatase inhibitors disrupts sexually motivated rhythmic cloacal sphincter movements, (Taziaux, Cornil & Balthazart, 2004). POM integrates this sensory and hormonal information and regulates these sexual behaviors. Here we argue that two efferent projections of POM are regulating different aspects of sexual behavior: (1) Previous studies demonstrated that POM projects to PAG, which in turn projects to the brain areas needed to implement the motor output required to engage in stereotypical consummatory sexual behaviors (Ball & Balthazart, 2004; 2010; Wild & Balthazart, 2013) (See figure 31). (2) The experiments reported in this dissertation demonstrate that the POM projects to the VTA and that these projections are involved in goal directed aspects of sexual behaviors. In mammals, dopaminergic projections from VTA to Ac are known to modulate incentive salience in association to a variety of rewarding stimuli (Berridge & Kringelbach, 2008). Potentially, the

functional link between POM and VTA facilitates the dopaminergic inputs to Ac which lead animals to rewarding stimuli. In mammals, one of the major outputs of the Ac is ventral pallidum (Vp), which is called the "limbic final common pathway" and is argued to be the converging locus for reward related signals (Smith et al., 2009). Vp is also documented to project to corticolimbic loops via its projections to medial prefrontal cortex to initiate more complex goal-directed responses to rewarding stimuli in mammals (Reviewed in Smith et al., 2009). In avian species, nidopallium caudolaterale (NCL) is considered to be the functional equivalent of the mammalian prefrontal cortex (Güntürkün, 2005) and it has been argued that it's involved in goal directed planned behaviors in pigeons (Starosta, Güntürkün, & Stüttgen, 2013). These circuitries have not been established in avian species, but a logical candidate for a neuroanatomical locus involved in the modulation of complex goal-directed behaviors associated with sexually rewarding stimuli would be the VP-NCL circuit. In mammals, Vp is also credited with contributing to the regulation of motor pathways involved in reward-related behaviors (Smith et al., 2009). Even though no experimental evidence has been collected, outputs from POM and Vp may be converging on motor pathways related to the implementation of stereotypical appetitive sexual behaviors. For example, projection of POM and Vp to dorsal PAG (formally known as intercollicular nucleus) may jointly influence crowing behavior in Japanese quail (see figure 30).

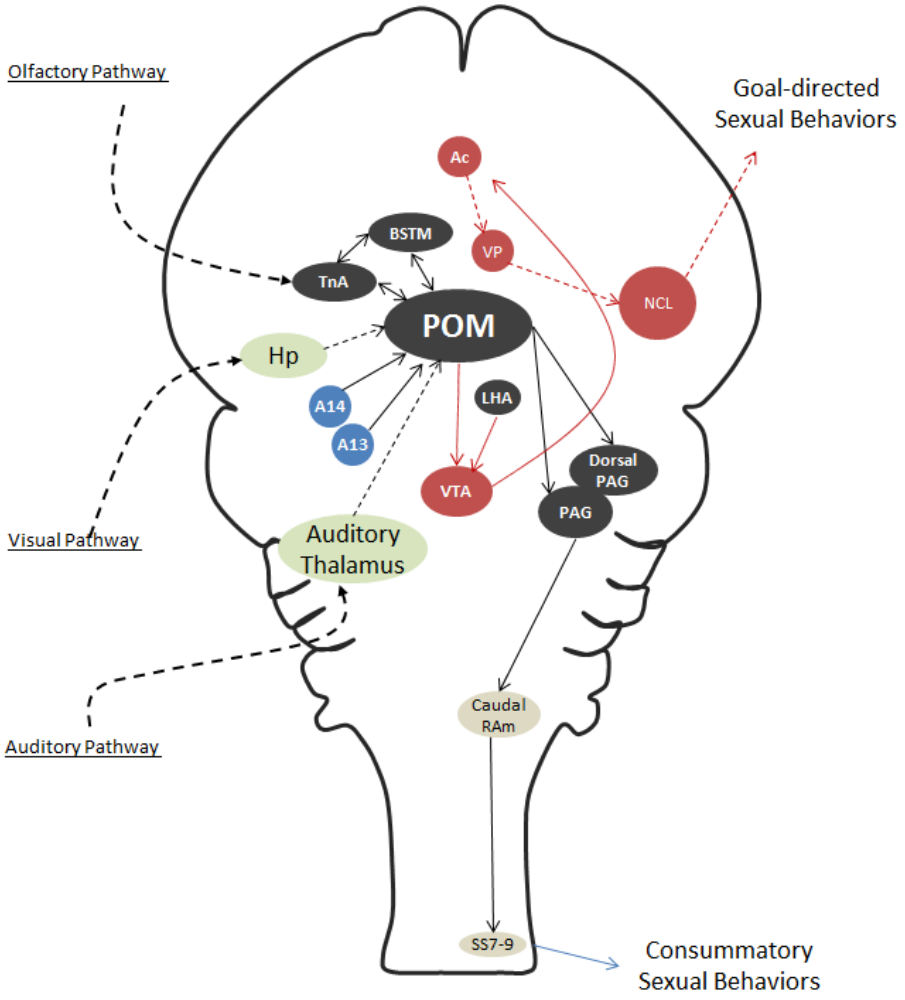

Figure 30. Diagrammatic representation of the putative interconnectivity of POM and mesolimbic reward circuitry in regulation of appetitive and consummatory sexual behaviors.

Concluding Remarks

From an evolutionary perspective, sociosexual interactions are fundamental for an organisms' reproductive success (Insel and Fernald, 2004). However, these interactions also have major costs, such as energy expenditure, risk of predation and disease that threatens the survival of the animal (Andersson, 1994). Furthermore, the appropriate social, environmental, and developmental context also influences the costs and benefits of the potential sexual interactions. Therefore, coordination of the sociosexual interactions under these complex conditions is

arguably the most important mediator for evolutionary fitness of the organism. The existence of these convoluted external and internal conditions necessitates an intricate interplay of different brain mechanisms addressing diverse problems to be able to respond in flexible ways. The present dissertation has provided novel evidence on how different brain mechanisms may interact in order to produce adaptively meaningful responses.

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Table of Figures

List of Abbreviations

The following table designates the various abbreviations and acronyms used throughout the dissertation.

Abbreviation	Meaning	Abbreviation	Meaning
3v	third ventricle	LoC	locus coeruleus
5-HT	5-hydroxytryptamine	M	mounts
6-OHDA	6-hydroxydopamine	MA	mount attempts
A10	ventral tegmental area	MDcp	dorsal corticoid plate region of mesopallium
A13	DA cells in zona incerta	Mst	medial striatum
A14	DA cells in periventricular hypothalamus	mPOA	medial preoptic area
ABC	Avidin Biotin Complex	MVcp	ventral corticoid plate region of mesopallium
Ac	nucleus accumbens	MVS	medioventral mesopallium
AcS	nucleus accumbens shell	NA	noradrenaline
AcC	nucleus accumbens core	NCL	nidopallium caudolaterale
ASB	appetitive sexual behavior	NG	neck-grab
AntC	anterior commissure	nPGi	nucleus paragigantocellularis
BNST	bed nucleus of stria terminalis	OD	optical density
BNSl	bed nucleus of stria terminalis lateralis	PAG	periaqueductal gray
BDA	biotinlylated dextran amine	PBS	phosphate-buffered saline
CSB	consummatory sexual behavior	PC	physical contact
CCM	cloacal contact movement	POA	preoptic area
DA	dopamine	POM	medial preoptic nucleus
DAB	3,3'diaminobenzidine tetrahydrochloride	PVN	paraventricular nucleus of the hypothalamus
Darpp-32	dopamine-and cAMP-regulated phosphoprotein, Mr 32 kDa	Ram	nucleus retroambigualis
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine	RCSM	rhythmic cloacal sphincter movements
DOPAC	3,4-Dihydroxyphenylacetic acid	Rp	raphe pallidus
Egr-1	early growth response protein 1	SBN	social behavior network
FCSM	female-typical cloacal sphincter movements	SCv	subceruleus ventrale
GABA	γ-Aminobutyric acid	SDM	social decision-making network
HC	holding cage	SERT	serotonin transporter
HA	hyperpallium accessorium	SS	synsacral segment
Hp	hippocampus	SN	substantia nigra
HPLC	high performance liquid chromatography	T	testosterone
IEG	immediate early gene	TH	tyrosine hydroxylase
IP	intraperitoneal	TPH	tryptophan hydroxylase
ir	immunoreactivity	TSM	tractus septopallio-mesencephalicus
IEG	immediate early gene	TnA	nucleus taenia of the amygdala
kDa	kilodalton	VC	visual contact
L-dopa	3,4-dihydroxy-L-phenylalanine	VL	lateral ventricle
LHA	lateral hypothalamus	VTA	ventral tegmental area
		VP	ventral pallidum

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PUBLICATIONS

Peer reviewed publications

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TALKS AND COLLOQUIA

İyilikci, O. On the Origins of Language. (Evolution of Human Sociality, Panel Chair: Hasan Bahcekapili). Bilgi University (2009), Dogus University (2008), Yeditepe University (2008).

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